NATURAL BIO-ANTIMUTAGENIC ACTIVITY OF ROOIBOS TEA (*Aspalathus Linearis*) AS EXPRESSED BY THE AMES, TOXI-CHROMO AND SOS-CHROMO TESTS

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DECLARATION

I, the undersigned hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

19.10.99

Lauren Standley Date

OPSOMMING

Rooibostee (*Aspalathus linearis*), 'n inheemse fynbosplant, word in die Wes-Kaap in die Clanwilliam omgewing verbou en het in gewildheid begin toeneem weens die beweerde gesondheidseienskappe daarvan. Die bevestiging van die antimutageniese potensiaal van Rooibostee is belangrik en navorsing op die chemo-voorkomende eienskappe daarvan behoort dit te onderskei as 'n drankie wat die gesondheid sal bevoordeel. Die antimutageniese eienskappe van gefermenteerde- en ongefermenteerde Rooibostee is bepaal en vergelyk met dié van groen- en swarttee (*Camellia sinensis*), onderskeidelik.

Die etielasetaat- en wateroplosbare fraksies van die vier tee ekstrakte is, onderskeidelik, getoets volgens die Amesmetode en *Salmonella typhimurium* TA 98 en die mutageen, 2-asetielaminofloureen (2-AAF), is gebruik. Die hoogste antimutageniese aktiwiteit is waargeneem met die groentee (67.6% inhibisie) gevolg deur die ongefermenteerde Rooibostee (59.1% inhibisie). Die gefermenteerde Rooibostee (47.3% inhibisie) en die swarttee (46.5% inhibisie) het die laagste antimutagenisiteit getoon. Die hoogste antimutageniese potensiaal is waargeneem met die etielasetaatfraksie, wat daarop dui dat meeste van die komponente verantwoordelik vir die tee se antimutagenisiteit moontlik in hierdie ekstrak voorkom.

Die ongefermenteerde Rooibos- en groentee wateroplosbare fraksies het dieselfde antimutagenisiese aktiwiteit getoon, indien gestandardiseer tot 'n standaard totale polifenolinhoud, terwyl die gefermenteerde Rooibostee 'n sterker antimutagenisiese aktiwiteit as swarttee getoon het.

Die Amestoets wat op monsters van onderskeidelik vyf prosesseringstappe van Rooibostee uitgevoer is, het daarop gedui dat prosessering die antimutageniese potensiaal van die tee verlaag. Resultate het bevestig dat die grootste verlaging in antimutageniese potensiaal tydens die fermentasie proses plaasgevind het.

Aangesien aspalatien een van die hoof polifenole in Rooibostee is, is die Amestoets uitgevoer op 'n ru-fraksie van aspalatien om te bepaal of polifenole of, meer spesifiek, aspalatien verantwoordelik is vir die antimutagenisiteit van Rooibostee. Die Amestoets het bewys dat die aspalatienfraksie antimutageniese

potential het, wat gemiddeld 5 - 15% laer was as dié van die wateroplosbare verbindings in Rooibostee.

Resultate van die Amestoets op kommersiële Rooibostee monsters van vyf verskillende kleinhandelaars het aangedui dat die vervaardigingsproses van Rooibostee, 'n produk met 'n redelike eenvormige antimutageniese potensiaal lewer.

Die SOS-Chromotoets en die Toxi-Chromotoets, wat onderskeidelik vir genotoksisiteit en vir toksisiteit toets, is op die vier wateroplosbare tee ekstrakte, naamlik ongefermenteerde Rooibos-, gefermenteerde Rooibos-, groen- en swarttee uitgevoer. Hierdie toetse is gebaseer op spesifieke mutante rasse van *Escherichia coli* en die resultate word bepaal deur kleurreaksies. Die SOS-Chromotoets kleurprofiel het swak ontwikkel, terwyl die kleurprofiele van die Toxi-Chromotoets aangedui het dat die tee ekstrakte moontlik 'n toksiese effek op die mutante bakterieë gehad het. Verdere ondersoeke is uitgevoer om hierdie onduidelike resultate te probeer verduidelik en groei studies, om te bepaal of die tee 'n inhiberende effek op die bakterieë het, is ook gedoen. Die resultate het aangedui dat ongefermenteerde- en gefermenteerde Rooibostee 'n baie sterk inhiberende effek op die groei van die bakterieë het, terwyl die groen- en swarttee 'n kleiner inhiberende effek op die groei van die bakterieë gehad het.

ABSTRACT

Rooibos tea (*Aspalathus linearis*), an indigenous fynbos plant, is cultivated in the Clanwilliam area of the Western Cape and has become increasingly popular due to its alleged health properties. The confirmation of antimutagenic potential in Rooibos tea is important and research on its chemo-preventive properties would substantiate it as a health promoting agent. The antimutagenic properties of fermented and unfermented Rooibos were determined and compared to that of green and black teas (*Camellia sinensis)*.

The Ames test, using *Salmonella typhimurium* TA 98 and the mutagen 2-acetylaminofluorene (2-AAF), was performed on the ethyl acetate and water-soluble fractions of the four tea extracts. The green tea had the strongest antimutagenic potential (67.6% inhibition), followed by the unfermented Rooibos tea (59.1% inhibition). The fermented Rooibos (47.3% inhibition) and black (46.5% inhibition) teas showed the lowest antimutagenic potential. The ethyl acetate extracts had the stronger antimutagenic potential, indicating that most compounds responsible for the teas antimutagenic potential are probably found in this extract.

At a standardised total polyphenol content the green (75.5% inhibition) and unfermented Rooibos (75.1% inhibition) teas had very similar antimutagenic potentials. The fermented Rooibos (73.0% inhibition) had a higher antimutagenic potential than the black tea (70.5% inhibition).

Ames tests done on samples from the five processing stages in the manufacture of Rooibos tea revealed that processing does cause a reduction in the antimutagenic potential of the tea. The major decrease in antimutagenic potential occurred after the fermentation stage ($P<0.05$).

Since aspalathin is one of the major polyphenols found in Rooibos tea, the Ames test was performed using a crude aspalathin fraction to determine if polyphenols or more specifically aspalathin, are responsible for the antimutagenic properties in Rooibos tea. The Ames test revealed that the aspalathin fraction had antimutagenic potential that, on average, was 5 - 15% lower when compared to the antimutagenic potential in Rooibos tea water-soluble extracts.

The results of the Ames tests performed, using *S. typhimurium* TA 98, on random Rooibos tea samples from five commercial retailers revealed that the processing of Rooibos tea produces tea with fairly uniform antimutagenic potential.

The SOS-Chromotest, to test for genotoxicity and the Toxi-Chromotest, to test for toxicity of the four tea extracts (fermented Rooibos, unfermented Rooibos, green and black tea), were also performed. These tests are based on mutant strains of *Escherichia coli* and depend on a colour reaction to determine results of the tests. Colour profiles from the SOS-Chromotest were too faint to determine results from, whereas the Toxi-Chromotest colour profiles indicated that the four tea extracts probably had a toxic effect on the mutant bacteria. Further investigations were conducted to try and explain these inconclusive results and growth studies to determine if the tea was having an inhibitory effect on the growth of the bacteria were performed on the bacterial tester strains and a standard *E. coli* culture (ATCC 58). The resulting data indicated that fermented and unfermented Rooibos had a very strong inhibitory effect on the growth of the bacteria while the green and black teas had lower inhibitory effects on the growth of the bacteria.

To my parents

CONTENTS

The language and style of this thesis are in accordance with the requirements of the *Journal of Agricultural and Food Chemistry*. This dissertation represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters, has, therefore been unavoidable.

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CHAPTER 1

INTRODUCTION

Teas are known to cause an increase in alertness, to diminish fatigue and to improve physical and mental performance. These beneficial characteristics are mainly ascribed to the high caffeine content of tea. Recently, other health benefits have been noted (Yen & Chen, 1995), including anti-depressive, anti-inflammatory, anti-hypersensitive, anti-athersclerotic and hypercholesteroteric properties. Teas are also good dietary resources for natural antioxidants such as polyphenolic compounds (Joubert & Ferreira, 1996).

Green tea, produced from the *Camellia sinensis* plant, has been consumed throughout the world for centuries (Wang *et al.*, 1989) and has shown antimutagenic properties to the *Salmonella typhimurium* strain used in the Ames test (Yen & Chen, 1995). Antimutagenicity is the ability of a compound to inhibit the process that leads to a change, or mutation in genetic material (Bronzetti, 1994). Polyphenols are the most abundant group of compounds in the fresh tea leaves and are believed to play an important role in the antimutagenic properties of green tea (Mukhtar *et al.*, 1992).

South African consumers and physicians regard Rooibos tea as beneficial to the body, improving the appetite, calming digestive disorders, reducing nervous tension and promoting sound sleep. In 1968, the Rooibos Tea Control Board was made aware of the case of a one year old infant allergic to milk, able to tolerate only a soybean formula, and suffering chronic stomach cramps, vomiting and restlessness. The child had failed to respond to normal medical treatment and weighed only 8.5 kg. After adding some Rooibos tea to speed up the warming of the soybean formula the child's symptoms improved and was given the same combination on a regular basis. She gained 2 kg in the next 10 days and recovered from her digestive trouble (Morton, 1983).

Rooibos tea, produced from the leaves of the *Aspalathus linearis* plant, contains polyphenols, no caffeine and has a low tannin content (Rabe *et al.*, 1994). Polyphenols are included in the "designer" food program of the National Cancer Institute (USA) where the safety of various phyto-chemicals

as chemo-preventive agents in experimental foods is tested (Caragay, 1992). This is an indication of their importance in cancer prevention (Ruch *et al.*, 1989; Hertog *et al.*, 1993; Price, 1994). The Japanese are known for testing physiologically active nutrients in food and drinks (Niwa *et al.*, 1991). They are already using Rooibos tea as an ingredient in a natural medicine as it is believed to play a role in relieving a wide variety of inflammatory diseases in patients and reducing their serum lipid peroxide levels (Niwa *et al.*, 1991). These beneficial effects of Rooibos tea are linked to its polyphenolic composition and associated antioxidant activity (Niwa & Miyachi, 1986).

It seems very unlikely that we will find cures for many types of cancer in the forseeable future. Therefore it would be a good idea to put considerable effort into cancer prevention, especially since there are reasons for believing that the vast majority of cancers are preventable. The frequency of different kinds of cancer varies enormously in different human populations. For example, breast and colon cancer statistics are much higher in the United States than in Japan, whereas stomach cancer is much higher in Japan. In these cases, diet is the most likely contributing factor. Liver cancer is highest in third world countries. Once again, diet seems the most likely cause. Lung cancer is more prevalent in countries where smoking is popular, and it is well documented that heavy smokers are more likely to get lung-cancer than non-smokers. In general it would appear that much of the variation in cancer incidence is associated with lifestyle and environment. Cancer trends tend to disappear from one generation to the next in migrant populations (Zubay, 1993).

There is a growing conviction that the carcinogenic agents in the environment are active cancer causing agents because they produce mutations in critical genetic elements within the cell (Maron & Ames, 1983). The search for carcinogens has been facilitated by Dr. Bruce Ames of the University of California in Berkeley, who developed a test for carcinogens based on the mutagenic action of a compound in specifically selected bacteria (Zubay, 1993). Ames tests, as designed by Bruce Ames (Ames *et al.*, 1975), have shown that many carcinogens originate from food and pollutants from the chemical industries.

Processing of the *Aspalathus linearis* plant to produce Rooibos tea involves, amongst other steps, a fermentation/oxidation process. This is triggered by cutting and bruising the tea between rollers. The distinctive colour, aroma and flavour of rooibos tea is released during the fermentation process (Morton, 1983). Aspalathin is oxidised during the process to form polymeric compounds and possibly the flavanones dihydro-2,3-orientin and dihydro-3,4-iso-orientin (Joubert & Ferreira, 1996). The composition of the tea leaves varies as the leaves proceed through the different processing stages. Optimisation of the different steps to retain maximum chemo-preventive properties without altering the flavour of the tea is of critical importance as it may contribute to the prevention of various types of cancer.

The aim of this research was to characterise the antimutagenic effects of Rooibos tea as well as green and black tea and to compare the antimutagenic properties of Rooibos tea from five processing stages. The genotoxic and toxic properties of Rooibos, green and black tea were also investigated.

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CHAPTER 2

LITERATURE REVIEW

A. BACKGROUND

Originating in China and South East Asia, tea has been cultivated and consumed for more than 2000 years. The Chinese value tea for its pleasant flavour and medicinal benefits, some of which have scientific interest today (Table 1). Tea was first introduced into continental Europe by the Dutch at the beginning of the sixteenth century and reached England and North America by the mid 1600's. Tea has become an important commercial product throughout the world with annual production of 2 610 569 metric tons in 1996 (Balentine *et al.*, 1997).

The introduction of tea into Western urban society in the seventeenth century was a major advance in healthy drinking (Marks *et al.*, 1997). At a time when water-borne diseases were rife, tea had a significant advantage over other beverages as it is prepared using boiling water. This was, however, a largely unrecognised and unintended benefit from tea drinking which gained popularity mainly because of it's flavour and stimulant properties. These stimulating properties contrasted to the inebriating properties of beer - one of the only drinks, apart from water, readily available to the public prior to that time. Beer was consumed in vast quantities during the 16th and 17th centuries in Britain and it is probably no coincidence that, with the increasing availability of tea as an alternative source of drink, the consumption of beer dropped progressively to reach it's minimum in the mid-twentieth century. Today tea is cultivated world-wide and used in every part of the globe (Marks *et al.,* 1997).

Tea is known to cause an increase in alertness, to diminish fatigue and to improve physical and mental performance. These characteristics have been recognised in China for centuries, and are mainly attributable to the caffeine content of tea. Other health benefits associated with tea have only recently attracted the interest of nutritionists in the USA and Europe (Yen & Chen, 1995). Four cups of tea with skimmed milk typically provide two thirds of the fluid humans need each day for

Table 1. Traditional health claims for tea (Balentine *et al.*, 1997).

Traditional Claims	**Possible scientific basis**
Improved blood flow	Vassodialation and decreased platelet activity
Elimination of alcohol and toxins	Increased activity of phase I and II enzymes
Clear urine and improve flow	Diuretic effects
Relieves joint pain	Anti-inflammatory activity
Improved resistance to diseases	Prevention of cancer and coronary heart disease

optimum health, along with 5% of the selenium, 25% of the riboflavin, 6% of the pento-thenate, 10% of the zinc, 10% of the folate, 9% of the potassium and 45% of the manganese humans are estimated to require, together with smaller but significant quantities of many trace nutrients (Marks *et al.,* 1997). However, this alone would not qualify tea for serious consideration as possessing positive health benefits - which many people now consider it to have.

Tea is considered to have a wide range of physiological properties. These include their being stimulants, anti-depressants, and anti-inflammatory, anti-hypersensitive, anti-atherosclerotic and hypercholesteroterric agents. Tea is also a good dietary resource for natural antioxidants known as polyphenolic compounds (Joubert & Ferreira, 1996; Wang *et al.*, 1989).

Antioxidants appear to have a protective effect against lipid peroxidation in humans which has been related to phenomena such as vascular disease, carcinogenesis, thrombosis and aging, among others (Duthie, 1991; Kinsella *et al.*, 1993; McCord, 1994). Information has been accumulated over the past years demonstrating their ability to scavenge radicals such as hydroxyl, superoxide and peroxyl radicals (Joubert & Ferreira, 1996).

Flavonoids are naturally occurring phenolic compounds in plants and their antioxidative properties have long been recognised. They have been reported to inhibit lipid peroxidation, to scavenge free radicals and active oxygen, to chelate iron ions and to inactivate lipoxygenase (Yen *et al.*, 1997).

There has been much interest in the use of polyphenols to protect against certain forms of cancer which includes the investigation of polyphenols as chemo-preventive agents by the National Cancer Institute (USA) (Joubert & Ferreira, 1996).

Japanese green tea has been shown to inhibit mutagenesis caused by carcinogens in experimental animals (Wang *et al.*, 1989). This property has been attributed to the presence of the many catechin compounds present in the tea. This phenomenon has not been properly investigated in Rooibos tea from the plant *Aspalathus linearis.* Rooibos tea is low in catechin compounds (Sasaki *et al.*, 1993) and it is therefore reasonable to expect that its mode of antimutagenesis may be different from that of Japanese green tea.

B. ROOIBOS TEA

B1. Introduction

Rooibos tea, South Africa's own indigenous tea (Rabe *et al.*, 1994), is becoming increasingly more popular due to it's alleged health properties. Rooibos tea is claimed to help improve appetite and cure insomnia, allergies and nervous complaints (Joubert & Ferreira, 1996). Rooibos tea is also considered as an "anti-aging" beverage by the Japanese who use it as an ingredient in a natural medicinal product believed to be effective in relieving a variety of inflammatory diseases as well as reducing serum lipid peroxide levels (Niwa *et al.,* 1988).

Dermatological disease studies have shown anti-viral and anti-inflammatory properties for Rooibos tea (Shindo & Kato, 1991). The antioxidant activity of Rooibos teas polyphenolic compounds may be responsible for this (Yoshikwa *et al.,* 1990; Ito *et al.,* 1991). Snyckers & Salemi (1974) attributed the anti-allergic property to the antispasmodic effect of quercetin, a flavonoid compound present in Rooibos tea. Interest has also been shown in the use of flavanols as agents which inhibit mutation and therefore help protect against various forms of cancer (Price, 1994). The unique polyphenolic composition of Rooibos tea may therefore provide the clue to some of its' apparent health properties.

B2. The agronomy of *Aspalathus linearis*

The Rooibos plant, *Aspalathus linearis,* is a leguminous shrub native to the Cedarberg mountains of the Western Cape in South Africa (Joubert, 1996). Rooibos grows there in it's natural state in an area of winter rainfall and coarse sandy soil (Morton, 1983). Attempts to grow it elsewhere in South Africa have not been successful. The plant belongs to the class *Rosaceae* (the rose family). *Aspalathus linearis* has slender stems and the leaves are linear and needlelike, 2 - 6 cm long (Morton, 1983). Rooibos tea is a beverage that is extracted from the processed leaves and stems of this plant.

The local inhabitants of the area were the first to discover that the fine needle-like leaves of the wild plant can be used as a tasty, aromatic tea. In 1904, Benjamin Ginberg, a Russian immigrant and pioneer in the area became involved in

Rooibos tea realising it's marketing potential (Morton, 1983). Today Rooibos tea is not only consumed by South Africans, but by an ever increasing number of people around the world. Rooibos tea is exported to countries such as Japan, Germany, Australia, New Zealand, Switzerland, Britain, the United States of America, South America, the Pacific Rim, the Far East and Southern Africa (Joubert, 1994). It's popularity in South Africa is illustrated by the fact that South African Rooibos tea drinkers consume on average six cups of Rooibos tea per day (Joubert & Ferreira, 1996).

B3. The production of Rooibos tea

In the past, the harvesting and processing of Rooibos involved gathering of plants in the wild and chopping the plants, bruising their stems and allowing them to ferment and to dry (Morton, 1983). Today, the plants are cultivated rather than collected from the wild, and vast improvements in processing technology and strict quality control have been introduced (Morton, 1983). Seeds are planted from February to March, and the plants are tended carefully for 18 months, when they are ready for the first harvesting. The harvest is bound, milled, drenched and then bruised between rollers to trigger the fermentation process. During the fermentation process the distinctive colour, aroma and flavour of Rooibos tea is released. The Rooibos tea is spread out thinly in the hot sunlight to dry, and then sucked up by special vacuum pumps before being delivered to the Rooibos processors (Morton, 1983).

At Rooibos (Pty) Limited it is graded in accordance with the corporation's standards. Rooibos tea material then undergoes a special process of pasteurisation and is dried once more over an air-bed drier. The Rooibos tea is finally weighed, packed and marketed under various brand names, either in tea bags or in loose leaf form (Dr E. Joubert, February 1999, ARC-Infruitec/Nietvoorbij, personal communication).

B4. Components of Rooibos Tea

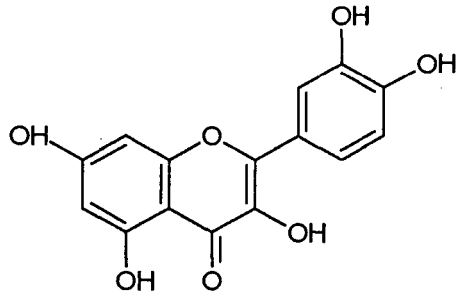
Rooibos tea is rich in volatile components, polyphenols and minerals (Rabe *et al.*, 1994). The major volatile components include guaiacol, damascenone, geranylacetone and phenylethyl alcohol (Habu *et al.*, 1985). Nothofagin, aspalathin, quercetin, iso-quercetrin and rutin are characteristic polyphenols found in Rooibos

tea (Fig. 1) (Joubert & Ferreira, 1996). The dihydrochalcone, aspalathin (2',3,4,4',6'-pentahydroxy-3-C-β-D-glycopyranosyldihydrochalcone) is the most abundant polyphenol found in unfermented Rooibos tea. It is unique to the tea and contributes significantly to the red-brown colour of the processed Rooibos tea (Koeppen & Roux, 1966). β-Hydroxy-dihydrochalcones are rare in nature and, apart from aspalathin, only pterosupin and nothofagin have been reported to be present in plant material (Joubert, 1996). Aspalathin is believed to undergo cyclisation to form dihydro-2,3-orientin and dihydro-3,4-iso-orientin, which are oxidised to form orientin and iso-orientin respectively during fermentation. Nothofagin, structurally similar to aspalathin except for the hydroxylation pattern of the B-ring (Fig. 1), is also present in unprocessed Rooibos tea. It was first isolated from the heartwood of *Nothofagus fusca*, at present the only other natural source of nothofagin (Joubert & Ferreira, 1996).

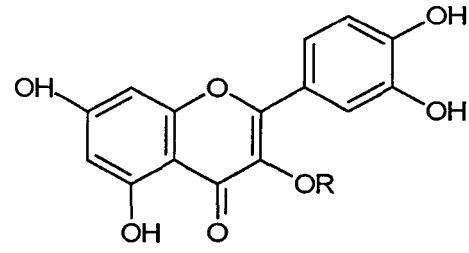
Other phenolic compounds present in Rooibos tea are the flavones which include iso-orientin, orientin, vitexin, iso-vitexin, chysoeriol and luteolin. Examples of phenolic acids in Rooibos tea include *p*-hydroxybenzoic, protocatechuic, vanillic, caffeic, *p*-coumaric and ferulic acids (Rabe *et al.*, 1994).

Flavonoids have a basic chemical structure consisting of a C_6-C_3-C_6 skeleton. Two aromatic rings are joined by an aliphatic three-carbon chain with hydroxyl, methoxyl or glycosyl groups attached at various points on the skeleton (Kühnau, 1976). They are naturally occurring, water–soluble substances found widely distributed in vegetables, fruit and beverages such as tea and wine. Red wine is a significant source of flavonoids in some cultures (Solomons & Bulux, 1994) and this contributes to the so-called "French Paradox". This describes a section of the French population which consumes a considerable amount of red wine and shows a reduced incidence of death as a result of heart disease in the presence of a high dietary intake of saturated fats (Kinsella *et al.*, 1993; Kondo *et al.*, 1994). In other cultures black tea, fruit and vegetables are the major source of dietary flavonoids (Hertog *et al.*, 1993).

Rooibos tea does not contain caffeine (Morton, 1983; Habu *et al.*, 1985). A number of physiological and pharmacological effects can be attributed to caffeine and arise mainly through its effect of blocking adenosine receptors. However, regular consumers of caffeine become tolerant to many of these effects. Caffeine



Quercetin

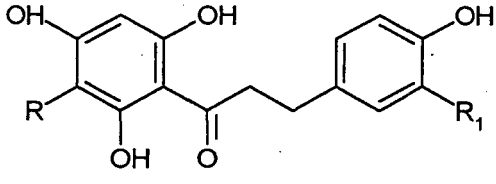


Iso-quercetrin

R = β-D-glucopyranosyl

Rutin

R = rutinosyl



Aspalathin: R_1 = OH

R = β-D glucopyranosyl

Nothofagin: R_1 = H

R = β-D-glucopyranosyl

Figure 1. Structure of characteristic polyphenols present in Rooibos tea (Joubert & Ferreira, 1996).

has a mild diuretic effect in some people, increasing the frequency of micturition but not increasing the urine volume. Caffeine is also a mild central nervous system stimulant and has broncho-dilatory properties. The potential relationship between caffeine and cardiovascular disease has long been the subject of research studies. Moderate caffeine consumption has not been associated with an increase in cardiovascular disease (Bimms, 1995).

Rooibos tea has a very low tannin content. Tannins are naturally occurring plant polyphenols. The small amounts present in Rooibos tea have not yet been defined (Rabe *et al.*, 1994).

C. OTHER TEAS

The tea plant belongs to the *Camellia* genus of the *Theaceae* family. Taxonomically speaking, two basic varieties of the tea plant are recognised, namely *Thea sinensis* and *Thea assamica.* The tea shrub is a perennial evergreen plant which yields crops throughout the year in tropical regions and for six to eight months in subtropical regions (Balentine *et al.*, 1997).

Tea, a hot water infusion of processed *Camellia sinensis* material, is consumed worldwide more than any other beverages except water (Stavric *et al.*, 1996). The reported beneficial effects of tea include: inhibition of carcinogenesis and mutagenesis; prevention of atherosclerosis; reduction of serum cholesterol; inhibition of platelet aggregation; and inhibition of nitrosamine reactions (Stavric *et al.*, 1996). Tea has been traditionally classified into six categories based on distinct manufacturing processes. These teas are green, black, dark green, oolong, white and yellow tea. The distinctive colour, flavour, and aroma of tea result from chemical changes that occur during leaf processing (Balentine *et al.*, 1997). Green teas are mostly consumed in Asian countries, while black tea products are more popular in Western countries. Green teas are generally non-fermented products processed in a manner designed to prevent enzymatic oxidation of catechins (Balentine *et al.*, 1997). Black teas are fermented products where browning reactions are catalysed by polyphenol oxidase. Catechins are reduced by 85% during black tea manufacturing, yet only 10% can be accounted for as theaflavins and theaflavic

acids. Theaflavins are a group of biopolymers that exhibit a bright orange-red colour in solution and contribute astringency and brightness to the tea beverage. Theaflavic acids are bright red, acidic substances present only in small quantities in black tea. Other water-soluble polyphenolic substances formed during the manufacturing process are thearubigens, thought to be the brown to black pigments of tea (Balentine *et al.*, 1997). Black tea beverage contains approximately 31% (m/m) flavonoids as 9% catechins, 4% theaflavins, 3% flavanols, and 15% undefined catechin condensation products. Green tea beverage contains approximately 33% (m/m) flavonoids as 3% flavonols and 30% catechins (Wiseman *et al.*, 1997). Dark green teas are fermented products in which the browning reaction is non-enzymatic. The oolong teas are semi-fermented products and the white teas are unfermented products produced from the tender leaves or unopened leaf buds. Yellow teas are made similarly to green teas except that they are slightly fermented, and non-enzymatic browning reactions occur to a lesser extent (Chen *et al.,* 1996).

C1. Green Tea

The term green tea refers to the product manufactured from fresh *Camellia sinensis* leaves in which significant oxidation of the major leaf polyphenols known as catechins is prevented (Ho *et al.*, 1994). For centuries green tea has been a widely consumed beverage throughout the world, particularly in parts of Asia. It is known to possess various beneficial pharmacological and physiological effects. These include antipyretic, diuretic, antioxidative and radioprotective activities (Wang *et al.*, 1989). A number of studies have shown that the death rate from cancer, especially stomach cancer, is lower in parts of Japan where green tea is a popular food product, compared with the rest of the population. Similar results may be cited for studies in other countries including China (Price, 1994).

The pharmacological effects of green tea are thought to be due to the presence of various polyphenols. Polyphenols are the most abundant group of compounds in fresh tea leaves and constitute ca. 30 - 42% of the total dry matter (Yen & Chen, 1996). Green tea polyphenols have been shown to inhibit the mutagenicity of nitrosation products of methylurea in *Salmonella typhimurium* TA 1535, and to reduce the number of high spontaneous mutations in a mutator strain of *Bacillus subtilis* (Wang *et al.*, 1989). In Table 2 some carcinogens and tumour

Table 2. Several physical and chemical carcinogens and tumor promoters that are broadly protected against by green tea and its constituents (Ho *et al.*, 1994)

I. **Indirect chemical carcinogens**

a. Benzo[*a*]pyrene (BP)

b. 7,12-Dimethylbenz[*a*]anthracene (DMBA)

c. 3-Methylcholanthracene (3MC)

d. 4-(Methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK)

e. *N*-Nitrosodiethylamine (NDEA)

f. Azoxymethane (AOM)

g. *N*-Nitrosomethylbenzylamine (NMzA)

II. **Direct chemical carcinogens**

a. *N*- Ethyl-*N*'-nitroguanidine (ENNG)

b. *N*-Methyl-*N*'-nitro-N-nitroguanidine (MNNG)

c. *N*-Methyl nitrosourea (MNU)

III. **Physical carcinogens**

a. Ultraviolet B (UVB) light

b. Ionising radiation

IV. **Tumor promoters**

a. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)

b. Teleocidin

c. Okadaic acid

d. Mirex

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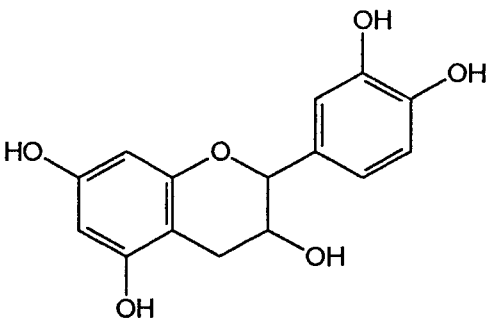
promoters are listed that are commonly used as research tools in the laboratory to study the effects of green tea and its constituents on the process of carcinogenesis *in vitro* and *in vivo* (Ho *et al.*, 1994).

In a series of mutagenicity assays done on green tea, oolong tea, black tea and pouchong tea, the green tea extract and the oolong tea extract exhibited the greatest inhibitory effect. Green tea contains the largest amounts of catechins and ascorbic acid compared to the other three tea extracts (Yen & Chen, 1995). Catechins are the major flavanols found in green tea. They are colourless, water-soluble compounds that contribute bitterness and astringency to green tea (Balentine *et al.*, 1997). The four most abundant catechins include: epigallocatechin gallate (ECGC); epigallocatechin (EGC); epicatechin (EC); epicatechin gallate (ECG) (Fig. 2) (Price, 1994). These compounds interact with specific enzymes (e.g. inducing selectively liver cytochrome *P*4501A1 and especially 1A2 and 2B1 in rats) which are thought to be responsible for activating food mutagens/carcinogens (Stavric *et al.*, 1996).

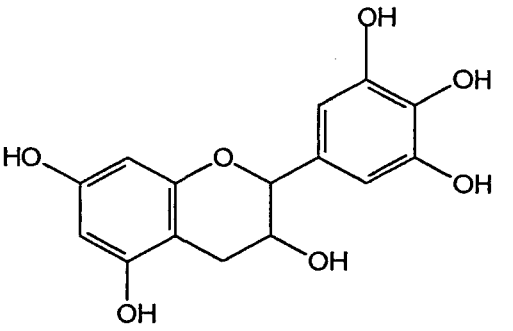
Catechins undergo oxidation to form theaflavins and thearubigens depending on the extent of fermentation (Xie *et al.*, 1993). According to Ho *et al.* (1994), unfermented green tea showed higher antioxidant activity than semi-fermented, oolong or black tea. Yen & Chen (1995), however, found that the antimutagenic effect of semi-fermented or oolong tea was stronger than unfermented or fully fermented tea. This suggests that some antimutagenic substances may be formed during the manufacturing process of tea.

D. PHENOLS AS ANTIOXIDANTS

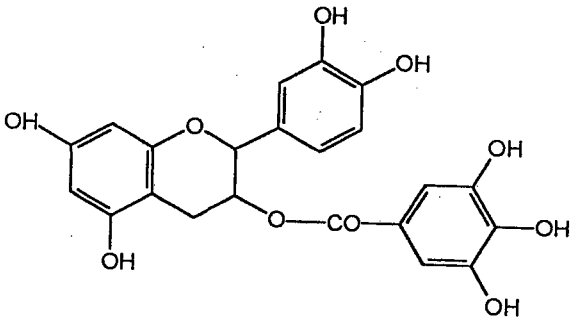
Normal metabolism of oxygen may result in the formation of the free radical superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Yen *et al.*, 1997). These compounds have the ability to generate the hydroxyl radical ($OH^\bullet$) *in vivo* in the presence of suitable transition metals e.g. iron (Halliwell *et al.*, 1992). The hydroxyl radical attacks different substances in living tissue (e.g. lipids, proteins and DNA) which may result in lipid peroxidation, protein denaturation and DNA mutation (Halliwell, 1987, Namiki, 1990). Radiation, air pollutants, tobacco smoke, pesticides, certain



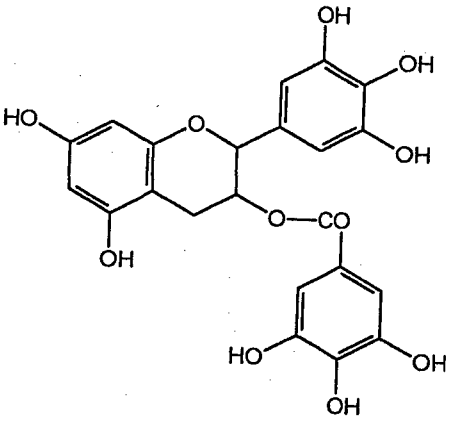
Epicatechin



Epigallocatechin



Epicatechin gallate



Epigallocatechin gallate

Figure 2. The most abundant catechins found in green tea (Price, 1994; Balentine *et al.*, 1997)

drugs, transition metals (iron) and alcohol are also sources of reactive oxygen species. Antioxidant enzymes and antioxidant nutrients such as vitamins A, C and E help protect the body against oxidative substances (Halliwell *et al.*, 1995). But if the radical defense mechanisms fail or are weakened, as a result of aging and inadequate nutrition, oxidative stress occurs. In severe cases oxidative stress can cause cell damage and death. Diseases such as atherosclerosis, inflammatory diseases (arthritis), heart disease, Alzheimer's disease, cancers and AIDS may be aggravated by irregular oxidative levels (Joubert & Ferreira, 1996).

According to a very general definition, antioxidants are "substances capable of delaying, retarding or preventing oxidation processes" (Schuler, 1990). The two groups of antioxidants are known as primary or chain breaking antioxidants and can react with liporadicals to convert them to more stable products, and secondary or preventative antioxidants which reduce the rate of chain initiation by various mechanisms (Gordon, 1990).

Several of the flavonoids found in Rooibos tea have shown antioxidant activity. These include: quercetin; luteolin; rutin; iso-quercetrin; and iso-vitexin. All flavonoids with the 3',4' -dihydroxy configuration possess antioxidant activity. The ene-diol functionality in the electron rich aromatic B-ring system could supply the electrons required for the reduction of the active oxygen species rendering them harmless (Joubert & Ferreira, 1996).

In the past, research has focused mainly on the antioxidant properties of β-carotene (provitamin A), vitamins C and E. Scientists are starting to appreciate the significance of other natural dietary substances such as flavonoids in cancer prevention and reduction of the frequency of heart disease. It is logical to assume that compounds occurring naturally in foods and that have been consumed for hundreds of years will be safer than synthetic ones. Allergic reactions and toxicity reports have been linked to synthetic antioxidants whereas natural antioxidants have not had the same negative side effects. The use of natural antioxidants in foods for stabilisation against oxidative changes is therefore gaining more acceptance as consumer resistance to synthetic antioxidants is increasing (Joubert & Ferreira, 1996).

E. CANCER AND IT'S CAUSES

Efforts in cancer chemotherapy have intensified over the past several decades, but cancers still remain difficult to cure. Cancer prevention could therefore become an increasingly useful strategy in the fight against cancer. Human epidemiological and animal studies have shown that the risk of cancer may be reduced by changes in dietary habits or dietary components. Humans ingest large numbers of naturally occurring antimutagens and anticarcinogens in food. These antimutagens and anticarcinogens may inhibit one or more stages of the carcinogenic process and prevent or delay the formation of cancer (Figure 3) (Ho *et al.*, 1994).

Cancer is the uncontrollable proliferation of certain of mammalian cells and is caused by mutations in the genetic material of the cells. Damage to DNA and cell division (which converts DNA lesions to mutations) are two critical factors responsible for the formation of mutations. Certain agents increase either the frequency of lesions or affect cell division. This can increase mutations or give rise to tumors and as a result increase cancer incidence (Henderson *et al.,* 1991; Cohen & Ellwein, 1992; Ames *et al.*, 1995).

Factors resulting in DNA lesions or cell divisions include: diet, smoking, aging, oxidative damage, chronic infection and hormones. Other factors include pollution and work space environments (Ames *et al.*, 1995).

E1. Oxidative damage and degenerative diseases of aging

The accumulation of oxidative damage to DNA and other macromolecules are largely responsible for aging and its degenerative diseases (Ames *et al.*, 1993). By-products of normal metabolism (superoxide, hydrogen peroxide and the hydroxyl radical) are the same oxidative mutagens produced by radiation (Vonn Sonntag, 1987) which may cause the DNA mutation. Oxidative lesions in DNA accumulate with age, so that by the time a rat is old (two years) it has about a million DNA lesions per cell, which is about twice that in a young rat. DNA is oxidised during normal metabolism when the antioxidative defenses present in the body (vitamins A and E and carotenoids) are depleted (Ames *et al.*, 1993).

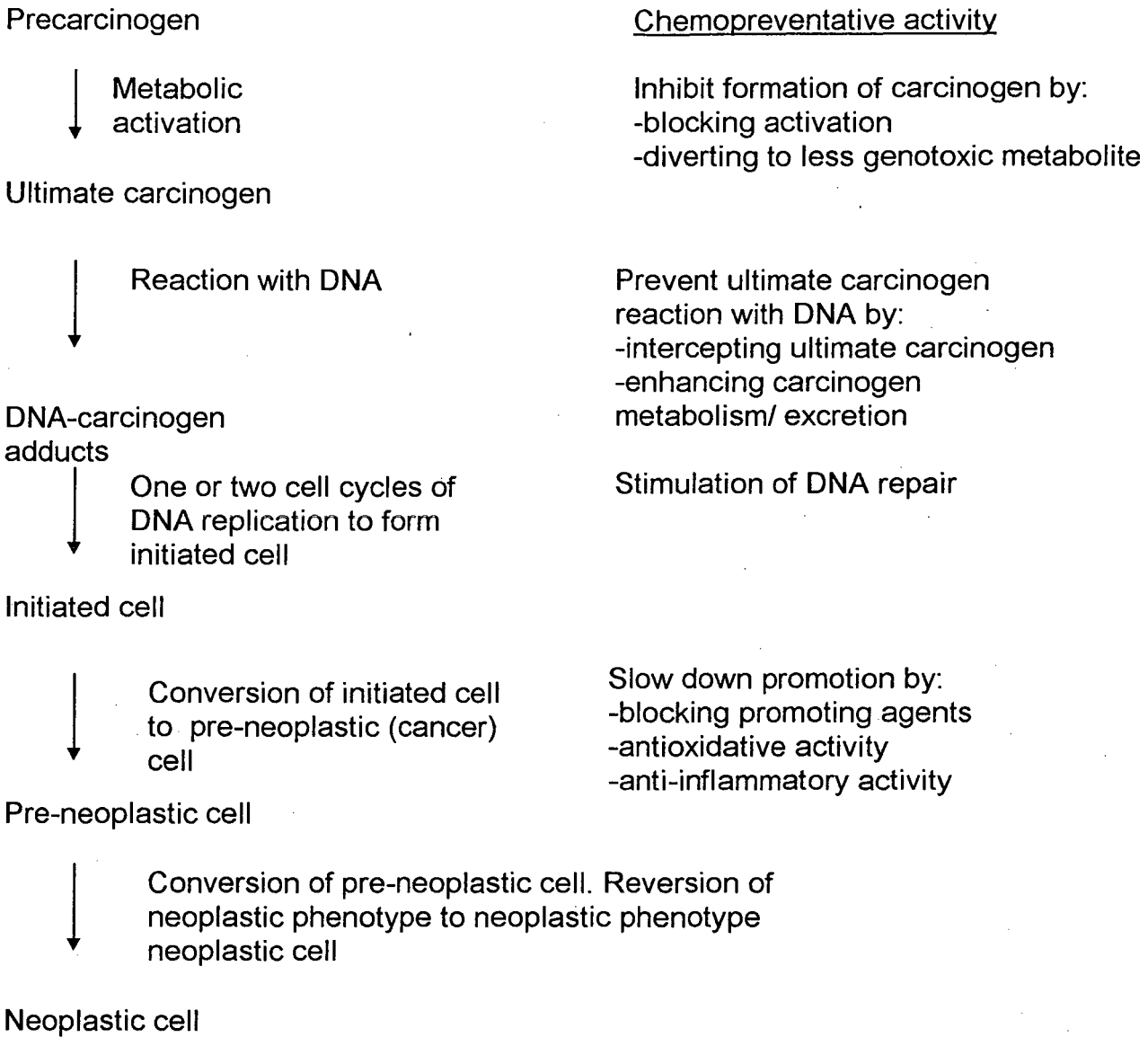


Figure 3. Multistage carcinogenesis model and possible points of intervention by chemopreventative agents (Ho *et al.*, 1994).

E2. Diet

Doll & Peto (1981) estimate that diet accounts for about one third of cancer risk in humans, and current research is aiming to clarify specific factors involved. Much research is being done on cancer prevention by dietary fruits and vegetables, as adequate consumption of fruits and vegetables is associated with a lowered risk of degenerative diseases, such as cancer, cardiovascular disease, cataracts and brain and immune dysfunction (Ames *et al.*, 1993). It has been reported that nearly 200 studies in epidemiological literature have been reviewed, and they show a consistent association between inadequate consumption of fruits and vegetables and cancer (Steinmetz & Potter, 1991a; Block *et al.*, *1992*; Hill *et al.*, 1994). For hormonally-related cancers, the protective effect of consuming fruits and vegetables is weaker and less consistent (Howe *et al.*, 1990; Block *et al.*, 1992; Hunter & Willett, 1993).

Laboratory studies suggest that antioxidants such as vitamins C and E and carotenoids account for the beneficial effects of fruits and vegetables (Ames *et al.*, 1995). However, the effects of other dietary components such as other vitamins and other ingredients in fruits and vegetables are not fully known (Steinmetz & Potter, 1991b; Block, 1992). A wide variety of compounds in fruit and vegetables in addition to antioxidants may contribute significantly to the reduction of cancer.

Research suggests that animal (but not vegetable) fat and red meat may increase the incidences of cancers of the breast and prostate (Armstrong & Doll, 1975). Mechanisms for these associations are not clear, but may include the effects of dietary fats on endogenous hormone levels (Henderson *et al.*, 1991), the local effects of bile acids on the colonic mucosa, the effects of carcinogens produced by cooking meat and excessive iron intake from red meat (Ames *et al.*, 1993).

E3. Tobacco

Tobacco is a known cause of cancer of the lung, bladder, mouth, pharynx, pancreas, stomach, larynx, oesophagus and possibly the colon (Fielding, 1994; Giovannucci *et al.*, 1994). Tobacco related diseases cause even more deaths than cancer (Ames & Gold, 1996). The evidence for environmental tobacco smoke as a cause of cancer is much weaker. Smoke contains a wide variety of mutagens. Oxidants in the smoke, mainly nitrogenoxides, deplete the body's protective antioxidants, resulting in damage to the DNA of the cells (Bui *et al.*, 1991).

E4. Cancer from inflammation caused by chronic infection

White cells and other phagocytic cells of the immune system combat bacteria, parasites and virus infected cells by destroying them with potent mutagenic oxidising agents. These oxidising agents may protect humans from immediate death from infection, but they also cause oxidative damage to DNA, mutation and chronic cell killing (Yamashina *et al.*, 1987; Shacter *et al.*, 1988). They therefore contribute to the carcinogenic process. Antioxidants appear to inhibit some of the pathology of chronic inflammation (Ames *et al.*, 1993). Examples of antioxidants include vitamins C and E and carotenoids.

E5. Hormones

In the extensive literature published on hormones and cancer it has been shown that the endogenous reproductive hormones play a large role in cancer (Preston-Martin *et al.,* 1990). It is possible that hormones contribute to as much as one third of all cancer, including breast (estrogen), prostate (testosterone), ovary and endometrium. Hormones are likely to act by causing uncontrolled cell division (Henderson *et al.*, 1991).

F. ANTIMUTAGENICITY

The process that leads to a change, or mutation in genetic material is known as **mutagenesis** (Bronzetti, 1994). The mutation could involve a single gene or a large portion of DNA. When mutagenesis occurs in the DNA of somatic cells, the mutation is restricted to the tissues of the affected organism. When the mutation occurs in the DNA of germ cells or their precursors, the mutation may be passed on to the later generations of the organism. As a result of damage to the DNA expression of proteins and enzymes in the cells may therefore also be affected. This may cause abnormal development in the offspring. Mutation normally occurs at a low rate, but this can be increased by the action of various physical or chemical agents, known as **mutagens**. Examples of mutagens include ionising radiation and ultra violet (UV) light as well as certain chemicals (Table 2).

Mutagenesis has been shown to have a role in the initiation stage of carcinogenesis. **Carcinogenesis** is a multi-stage process that leads to the development of cancerous tumours (Bronzetti, 1994). However, not all compounds that have been shown to induce carcinogenesis in epidemiological studies in humans are able to induce mutagenesis in short-term tests (such as the Ames test, SOS-Chromotest and the Toxi-Chromotest, (Bronzetti, 1994).

Antimutagens are various compounds that are able to decrease or inhibit the effect of mutagens (Bronzetti, 1994). Antimutagens can be divided according to their mechanism of action into two main classes i.e. desmutagens and bioantimutagens (Fig. 4). **Desmutagens** are compounds that act directly on mutagens or their precursors, mainly by chemical or enzymatic mechanisms, inactivating them before they are able to damage DNA. In the majority of cases, the inhibition occurs outside the cell (Price, 1994). **Bioantimutagens** in contrast are compounds that reduce the effects of mutagens by modulating cellular mutagenic processes. They act mainly on DNA replication and repair processes, reducing the extent of DNA damage that has been induced by mutagens (Bronzetti, 1994).

The human diet contains a variety of natural mutagens and carcinogens, as well as a variety of antimutagens and anticarcinogens. The body normally produces several enzymes such as superoxide dismutase, glutathione peroxidase and glutathione transferase (Bronzetti, 1994) that protect cells from oxidative damage induced by oxygen free radicals. The diet contains a wide variety of other compounds (Table 3) that have antimutagenic properties, many of these compounds appear to act as antioxidants. β-carotene is one of a class of lipid soluble pigments (the carotenoids) found in foods, fruit and vegetables. β-carotene can be enzymatically hydroxylated to form two molecules of vitamin A and it is therefore an important provitamin and is considered to be a potent natural antioxidant. β-carotene is fat soluble and protects against oxidation and similar polyprenes are present in foods containing chlorophyllin, such as carrots (Bronzetti, 1994). Vitamin E is considered to be one of the most important dietary components contributing to the body's antioxidant defence system. Vitamin E is a collective term, encompassing eight different products synthesised by plants, which can be divided into two classes, the tocopherols, which have saturated side chains, and the tocotrienols which have

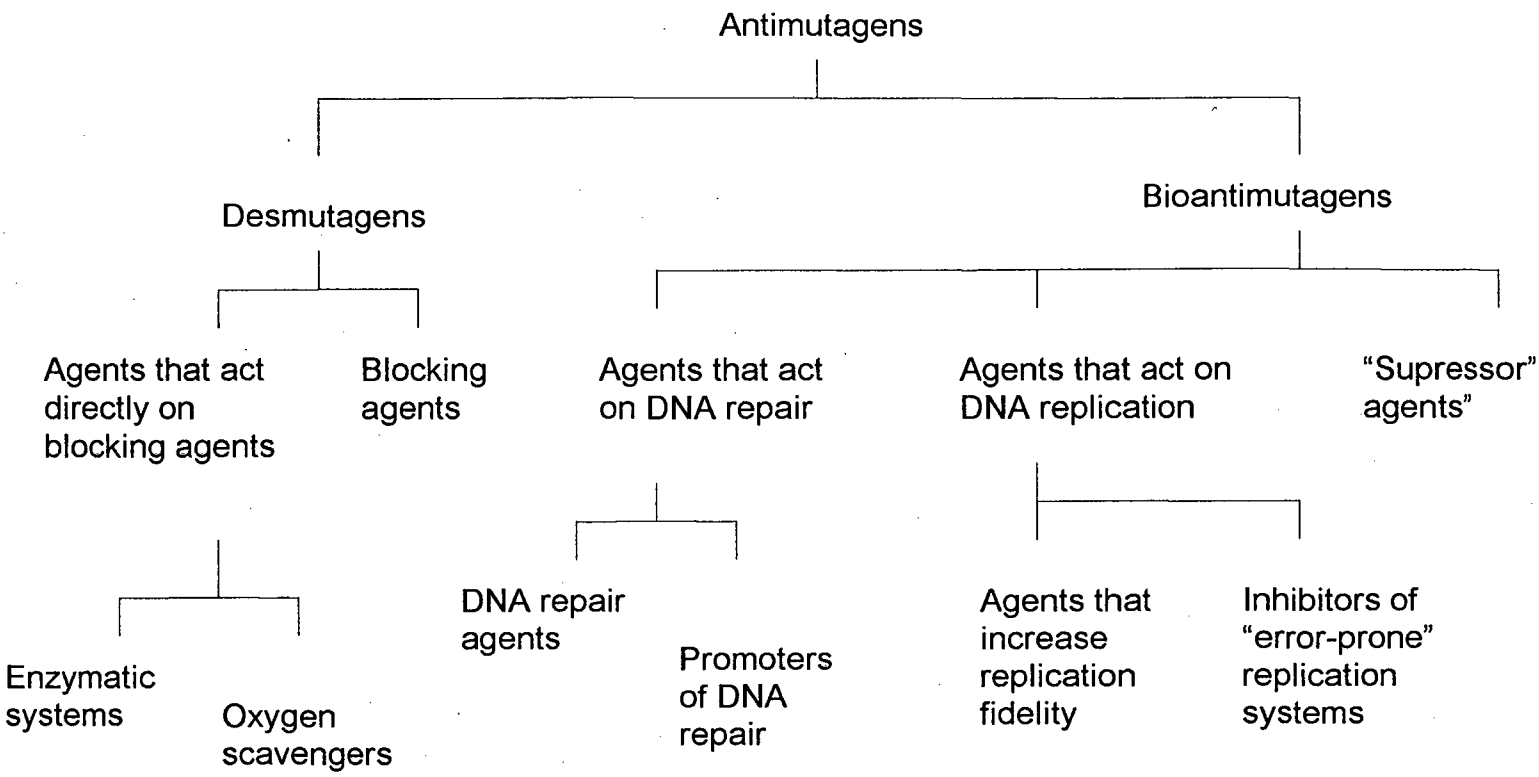


Figure 4. Postulated mechanisms by which antimutagens exert their effects (Bronzetti, 1994).

Table 3. Summary of some antimutagens/anticarcinogens found in foods (Bronzetti, 1994).

Food or Component	Antimutagen	Anticarcinogen	Mechanism
β-carotene (fresh fruit & vegetables)	Yes	Yes	Antioxidant
Vitamin E (plant oils & green leafy vegetables)	Yes (desmutagen)	Yes	Antioxidant, nitrite scavenger
Glutathione (animal cells)	Yes (desmutagen)	Yes	Antioxidant
Vanillin (used in flavouring foods)	Yes (bioantimutagen)	ND	DNA repair
Cinnamaldehyde (flavouring agent in confectionary & beverages)	Yes (bioantimutagen)	ND	DNA repair, DNA replication
Vitamin C (fresh fruit & vegetables)	Yes (desmutagen)	Yes	Nitrite scavenger
Miscellaneous fruit and vegetables	Yes (mainly desmutagens)	ND	Nitrite scavengers
Chlorophyllin	Yes (biomutagen or desmutagen)	ND	Antioxidant
Fermented milk	Yes	ND	Nitrite scavenger
Milk alone	ND	Yes	Nitrite scavenger
Lactobacilli alone	Yes	Yes	Nitrite scavenger
Garlic	Yes	Yes	Antioxidant
Diallyl sulphide (principal thioether of garlic)	Yes	Yes	Unknown
Selenium[a]	Yes	Yes	Antioxidant
Magnesium (milk)	ND	Yes	Unknown

[a]May also act as a mutagen at some concentrations
ND Not determined

unsaturated side chains. The anticancer properties of tocopherols are related to their ability to inhibit the auto-oxidation of unsaturated fatty acids. Vitamin E is fat soluble, and the richest natural sources are plant oils (e.g. wheat germ, rice, cottonseed and the lipids of green leafy vegetables) (Bronzetti, 1994).

The consumption of milk has also been shown to reduce the incidence of human stomach cancers induced by alkylating agents. It is well known that vegetables such as cabbage, ginger, spinach, lettuce and cauliflower have antimutagenic activity (Bronzetti, 1994). It has been suggested that vitamins, fiber and other components of vegetables act primarily as desmutagens. Other examples of compounds with antimutagenic activity are shown in Table 3.

There are three fundamentally different types of carcinogenic screening tests, based on whole animals, intact cells and subcellular preparations. The most direct approach is to screen for activity *in vivo* using an animal model. However, the use of *in vivo* screens is severely restricted due to the limited number of samples that can be handled, due to excessive cost and the relative insensitivity of the screen (*Ames et al.*, 1993). For this reason, only the latter two categories will be considered for screening Rooibos tea for antimutagenic properties.

F1. The Ames test

Chemical carcinogenesis, or cancer induced or aided by a chemically defined substance, is of great current interest in scientific investigation. This interest has been fuelled by studies of the past two decades indicating that the causes of a few types of human cancers (e.g. esophageal cancer and lung cancer) may be related to the environment. The chemical composition of the food consumed, the air breathed and the fluids drunk, is intimately involved with the carcinogenic process (Ames *et al.*, 1995).

Diets with a high vegetable content has been repeatedly correlated with reduced human cancer incidence at certain sites (Graham *et al.*, 1978). This correlation has led to the evaluation of various plant compounds as possible anticarcinogens, that may contribute to reduced cancer risk (Ames *et al.*, 1995). The most extensively studied compound is ellagic acid, which was shown to inhibit the mutagenicity of a number of promutagens and ultimate mutagens in the Ames test (Ames *et al.*, 1995). There is considerable evidence, most of it obtained using the

Ames test, that with few exceptions, carcinogens are mutagens. So far 85% of the carcinogens tested have been detected as mutagens (Ames *et al.,* 1995) whereas less than 10% of non-carcinogenic compounds act as mutagens. This establishes a rather high correlation between these two properties and supports the idea that a significant proportion of mammalian cancers may arise as a consequence of induced somatic mutations (Gerhardt *et al.*, 1981).

In 1974, Dr. Bruce Ames developed the "Ames test" for mutagenicity at the University of California in Berkeley. Dr. Ames' research focused on the oxidative damage to DNA and its relationship to mutagenesis, carcinogenesis and the degenerative diseases of aging (Ames & Gold, 1996).

The Ames test measures the rate of reversion of histidine auxotrophs of *Salmonella typhimurium* to prototrophy in both the presence and absence of the chemical being tested (Ames *et al.*, 1975). If the chemical is mutagenic (it will cause a change or mutation in DNA material) and by implication carcinogenic, it will increase the reversion rate. The reversion rate refers to the number of bacterial cells of *Salmonella typhimurium* that revert to histidine prototrophs. These cells do not have structural mutations in the structural genes for histidine biosynthesis as the original histidine auxotrophs have. The mutagenic chemical therefore caused a structural change in the cell's DNA. The Ames test also gives an indication as to the potency of a mutagen and therefore how hazardous a chemical is by the number of revertants that arise (Nester *et al.*, 1973). The more revertants that arise, the more powerful the mutagen. The test is based on the rationale that a change in the DNA of a mutant can result in a cell that resembles the original, non-mutant cell. The test is based on the reasonable premise that mutagenesis and cancer induction both result from alteration of the DNA of a cell (Nester *et al.*, 1973).

Spontaneous reversion of the tester strains to histidine dependence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants per plate. A compound is mutagenic when the revertant colonies are at least three times that of the spontaneous reversion rate. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. There is, however, variability in the number of spontaneous revertants from one experiment to another and from one plate to another. It is therefore advisable in a single experiment to perform all tests in triplicate (Maron & Ames, 1983).

The Ames test involves combining the test compound, the bacterial tester strain, and S9 mix in soft agar which is poured onto a minimal agar plate. Positive and negative controls are also included in each assay. After incubation at 37°C for 48 hours, revertant colonies are counted (Ames *et al.*, 1995). Many carcinogens and mutagens require metabolic activation before exerting their carcinogenic and mutagenic activities. The Ames test meets this requirement by including the S9 mix (post-mitochondrial extracts of mammalian liver) into the assay mixture (Gerhardt *et al.*, 1981).

The histidine auxotrophs of *Salmonella typhimurium* are known as the tester strains and each carry specific mutations in one of the structural genes for histidine biosynthesis, including frameshift and base pair substitution mutations. In addition to the histidine mutation, the standard tester strains contain other mutations that greatly increase their sensitivity to detect mutagens. One mutation (*rfa*) causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria and increases permeability to large molecules such as benzo[*a*]pyrene that do not readily penetrate the normal cell wall (Maron & Ames, 1983). The other mutation (*uvr B*) is a deletion of a gene coding for the DNA excision repair system, resulting in greatly increased sensitivity in detecting many mutagens (Maron & Ames, 1983). Other strains containing the R factors (plasmid determining drug resistance) have been developed which further enhance the detection of mutagenicity for several classes of mutagens (Maron & Ames, 1983). Different strains are used to detect different mutations e.g. *Salmonella typhimurium* TA 98 is used to detect frameshift mutations, TA 100 is used to detect base pair substitution mutations and TA 102 is used to detect oxidative damage.

The Ames test has been used to test the mutagenicity of many chemical compounds. Examples of compounds, which have been tested, include alkylating agents, nitrosamines, polycyclic hydrocarbons, fungal toxins, aromatic amines, nitrofuran carcinogens, a variety of anti-neoplastic agents, and antibiotic carcinogens such as adriamycin, daunomycin and mitomycin. In addition, the known human chemical carcinogens that have been tested, are positive. These include β-naphthylamine, benzidine, cigarette smoke condensates, bischloromethylether, aflatoxin B, vinyl chloride and 4-aminobiphenyl (Maron & Ames, 1983).

The contribution of phenols, quinones and reactive oxygen species to the mutagenicity of white grape juice has been tested using the Ames test. It was found that polyphenol oxidase-catalysed oxidation of polyphenolic compounds generates genotoxic species that are, at least partly, responsible for the mutagenicity of grape juice (Patrineli *et al.*, 1997). Strains of *Salmonella typhimurium* have been used to determine the antimutagenic activity of water extracts of black and oolong tea as well as the antimutagenicity of green tea polyphenols. All of the tea extracts exhibited antimutagenic affects. Regardless of the difference in components between green and black tea, the two types of extracts had almost the same antimutagenic activity (Yamada & Tomita, 1994; Yen & Chen, 1996; Stavric *et al.*, 1996).

The effects of curcumin, the yellow pigment of the spice turmeric (*Curcuma longa*), on the mutagenicity of several environmental mutagens were also investigated using the Ames test. Curcumin inhibited the mutagenicity of cigarette smoke condensates, tobacco and benzo[a]pyrene in a dose-dependant manner. The observations indicated that curcumin may alter the metabolic activation and detoxification of mutagens (Nagabhushan *et al.*, 1987).

The antimutagenicity of polyphenol-rich fractions from *Sorghum bicolor* grain was also investigated with the Ames test. It was found that the crude polyphenols all acted as antimutagens when co-incubated with *Salmonella typhimurium* and standard mutagens (Grimmer *et al.*, 1992).

F2. SOS-Chromotest

The SOS-Chromotest is a quick, convenient and streamlined method for the determination of genotoxicity in a variety of samples. It is based on a novel engineered strain of *Escherichia coli* patented by the Institute Pasteur and licensed to Orgenics Ltd. (Fish *et al.*, 1987).

The capacity of the Ames test to identify carcinogens is higher than that of the SOS-Chromotest. However, because the number of false positive compounds was lower in the SOS-Chromotest, the specificity, i.e. capacity to discriminate between carcinogens and non-carcinogens of the SOS-Chromotest, appeared higher than that of the Ames test. The results of the SOS-Chromotest and of the Ames test can therefore complement each other (Quillardet & Hofnung, 1993).

Genotoxins are toxins that affect the genetic material of cells. They are unique among toxic substances as the result of their action on multicellular organisms and may not become obvious for a long time after exposure. They may only affect later generations of the organism. Because of this, it is important to be aware of the "hidden" threat and be able to detect it and it's sources efficiently (Fish *et al.,* 1987). Tests for DNA damaging agents using living organisms are rather slow and expensive. Ames *et al.* (1975) were the first to realise the association between DNA damage, mutation and cancer. They used the short life span of the *Enteriobacteriaceae* to imitate the longer life time of mammals in detecting DNA damage and DNA damaging agents (Fish *et al.,* 1987). All bacterial short term tests consist of two components i.e. the target bacterial cells and the metabolising system. The metabolising system is used to mimic the metabolism of the compound in mammalian cells. It is supplied exogenously, although the bacterial cell often has its own way of metabolising the compound tested. The compound to be tested is therefore incubated with these components and a response is monitored (Quillardet & Hofnung, 1993).

A genotoxic agent often causes a variety of lesions. Depending on its nature and position (particularly regarding the replication fork) each lesion may lead to at least four types of consequences which are neither mutually exclusive nor inclusive of other consequence:

i. The lesion may be processed without mutagenic effects. This may occur by 'reversion' or 'excision' of the lesion;
ii. The lesion may provide direct miscoding;
iii. The lesion may induce the SOS response that includes SOS mutagenesis;
iv. The lesion may induce other specific responses such as the adaptive response to alkylating agents, the response to oxidative agents, the heat-shock response (Quillardet & Hofnung, 1993).

The SOS response is important in the response of *Escherichia coli* to genotoxic agents because of its large spectrum of responses towards a wide variety of agents. Triggering of the system can thus be used as a general and early sign of DNA damage. Two genes play a key role: *lex A* encodes a repressor for all genes of the system; and *rec A* encodes a protein able to cleave the Lex A repressor upon

activation by a SOS inducing signal. The exact nature of the SOS inducing signal is not known but it is produced when DNA lesions 'perturb' or stop DNA replication (Quillardet & Hofnung, 1993).

Monitoring the triggering of the SOS system is performed by monitoring the expression of a SOS gene by means of a fusion with *lac Z*, the structural gene for *Escherichia coli* β-galactosidase. The SOS-Chromotest makes use of a *sfiA::lac Z* operon fusion. *Sfi A* is one of the SOS genes. The assay consists of incubating the tester strain with increasing concentrations of the agent to be tested. After allowing time for protein synthesis, β-galactosidase activity is assayed by a simple colorimetric assay.

The *Escherichia coli* PQ37 tester strain used in the SOS-Chromotest carries a *sfiA::lac Z* fusion and has a deletion of the normal *lac* region so that β-galactosidase activity is strictly dependent on sfi A expression. In addition, the strain is made genetically more susceptible to genotoxic agents. It is devoid of the excision repair pathway (uvr A mutation) so that a number of lesions are not, or slowly processed, and it has a mutation (*rfa*) which renders the cell envelope more permeable to a number of compounds (Quillardet & Hofnung, 1993).

The SOS-Chromotest has been used for various purposes such as the detection of beverage or food genotoxic contaminations. These include the determination of aflatoxin B1 in orange juice or cow milk (Quillardet & Hofnung, 1993), chlorinated butenoic acids in chlorinated drinking water (Tinkanen & Kronberg, 1990) and the monitoring of natural genotoxic compounds in smoked foods in reaction with nitrite under acidic conditions as they are suspected to be formed in the human stomach through an interaction between nitrite and suitable amine precursors (Quillardet & Hofnung, 1993). Other examples include detection of genotoxic pollutants in the aquatic environment, the influence of algae on the genotoxicity of industrial effluents and the verification of the absence of genotoxic activity of extracts from plants of medicinal importance (Vargus *et al.*, 1990).

There is growing interest in the field of antimutagens, anticarcinogens and antigenotoxic compounds and the SOS-Chromotest can be a useful system to search for new compounds which reduce the effect of genotoxins. Generally, screening of antigenotoxic compounds with the SOS-Chromotest consists of

measuring the inhibition by the compound to be tested by the SOS induction, triggered by a known genotoxic compound (Quillardet & Hofnung, 1993).

The whole test, including the revival of the bacteria, can be completed in six to seven hours (Fish *et al.*, 1987). Due to its easily detectable endpoint (the development of colour) and its indifference to microbial and nutrient contamination, the SOS-Chromotest seems to be most suitable for industrial development to a field operable, stable and diagnostic type assay.

Research done by Quillardet & Hofnung (1993) showed that for 452 compounds the results obtained in the SOS-Chromotest can be compared to those obtained in the Ames test. It was found that 373 (82%) of these compounds give similar responses in both tests (236 positive and 137 negative responses). Thus, the discrepancies between both tests concern 79 compounds (18%) (Quillardet & Hofnung, 1993). A case by case analysis showed that many of these compounds are at the same time very weak SOS inducers and very weak mutagens. It is thought that the discrepancies between the two tests may be accounted for by differences in the interpretation of the results rather than the experimental results themselves (Quillardet & Hofnung, 1993). However, there are some compounds (quinolene-1-oxide) which are clearly SOS inducers but devoid of mutagenic activity in the Ames test, as well as mutagenic compounds which do not induce the SOS response in the SOS-Chromotest (benzidine, ethidium bromide, cyclophosphamide) (Quillardet & Hofnung, 1993).

F3. The Toxi-Chromotest

In the modern world, industry plays a large role and as a result, numerous chemicals are being introduced. Many of them pose a problem as their release into the environment affects public health (Reinhartz *et al.*, 1987). Conventional toxicity bioassays using animals, and especially mammals, are time consuming and expensive. The Toxi-Chromotest is a bacterial test suitable for the detection of toxicants in water, chemicals, pharmaceuticals, foodstuffs, food additives and cosmetics. It is a simple, rapid (1 - 1.5 h), colorimetric assay which does not require special equipment, and can easily be carried out under field conditions. The microbial test has the advantage of being rapid because of the short life cycle of

bacteria. Bacteria are also simple to handle, their cultivation is cheap and rapid, and one can use very small volumes in the test.

The Toxi-Chromotest was developed by Orgenics Ltd. (Reinhartz *et al.*, 1987) and has a colorimetric end-point so that the results can be read with the naked eye or by using the microtitration plate photometers (ELISA readers). The test is based on a mutant strain of *Escherichia coli*. Toxicants easily penetrate the rough lipopolysaccharide cell wall and inhibit the synthesis of an inducible enzyme (e.g. β-galactosidase). Sensitivity of this bacterial strain to the toxicants is enhanced as the bacteria are pre-stressed by various methods (Reinhartz *et al.*, 1987).

In the Toxi-Chromotest the stressed bacteria are mixed with a cocktail containing a specific inducer of a bacterial enzyme, and essential factors required for the recovery of the bacterium from its stressed condition. The activity of the enzyme is detected by its effect on a chromogenic substrate. During the dehydration stage, structural and functional damage occurs. Following lypophilisation, *Escherichia coli* cells need a recovery period of about 30 minutes before their capacity to synthesise the enzyme (β-galactosidase) is re-established. This delay enables the toxin action to occur before β-galactosidase is synthesised, resulting in an increased sensitivity and lower background activity. This therefore interferes with the colour reaction that would be formed if β-galactosidase was produced. The colour reaction may be measured visually or spectrophotometerically (Kwan & Dutka, 1992).

In short the Toxi-Chromotest has three main advantages:

i. The increase in permeability of the bacteria during rehydration which increases sensitivity of the system towards otherwise non-penetrating toxicants;

ii. The increase in the number of metabolic and physiological functions essential for recovery from stress and enzyme induction. All, or any one of these functions may be sensitive to inhibition by the toxicants, thereby enlarging the spectrum of toxicants that may be detected; and

iii. The separation of the period of toxicant activity from the maximum rate of induction of β-galactosidase (occurring at the end of the recovery process)

which enables a maximal toxic effect prior to induction and the development of the colour signal in a one-step procedure (Reinhartz *et al.*, 1987; Kwan & Dutka, 1992).

F4. Advantages of the Toxi-Chromotest and the SOS-Chromotest over vertebrate/invertebrate screening tests

The Toxi-Chromotest and the SOS-Chromotest are advantageous over the traditional methods as they are simple to carry out and no special training is required. No special instrumentation is needed as only test tubes are needed and a visual quantitative colorimetric response is obtained within a few hours. The Toxi-Chromotest and SOS-Chromotest kits (Environmental Bio Detection Products Inc., Ontario, Canada) contain most of the materials necessary to perform the assay, and come with lists of the chemicals that must still be obtained. The tests are quick to perform (less than four hours) and are sensitive to low levels of toxicants or genotoxicants. They are reliable tests but are relatively expensive.

G. GENERAL DISCUSSION

Cancer is a disease that has affected millions of people around the world. The investigation to determine if Rooibos tea does have antimutagenic properties can only be beneficial in the search for a means to prevent cancer. The evidence that the polyphenols in green tea have antimutagenic properties suggests that Rooibos tea will also probably have these properties, as polyphenols are a major component of Rooibos tea.

Polyphenols are naturally occurring compounds in *Aspalathus linearis.* There is therefore little chance of toxicity or allergic reactions occurring as might occur with synthetic compounds. If the polyphenols do possess antimutagenic activities they may later be used in food products as food preservatives to help prevent mutagenic changes from occurring in the mammalian cells. They may also be used in nutraceuticals or designer foods.

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CHAPTER 3

DETECTION OF THE ANTIMUTAGENIC POTENTIAL IN FERMENTED AND UNFERMENTED ROOIBOS, GREEN AND BLACK TEAS USING THE *SALMONELLA TYPHIMURIUM* MUTAGENICITY (AMES) TEST

Abstract

The antimutagenic potential of the aqueous extracts of four teas was determined using the *Salmonella typhimurium* mutagenicity (Ames) test. These extracts included fermented and unfermented Rooibos (*Aspalathus linearis*), green and black (*Camellia sinensis*) teas. The total soluble solid and flavonoid content of each extract was determined and it was found that green tea had the highest soluble solid (3.0%) and flavonoid content (51.7 mg.100 ml^{-1}) and fermented Rooibos tea the lowest with 1.3% soluble solids and 25.6 mg.100 ml^{-1} flavonoids. Although unfermented Rooibos tea had a lower soluble solid content (1.8%) it had a higher flavonoid content (40.7 mg.100 ml^{-1}) than the black tea (35.5 mg.100 ml^{-1}). The mutagen 2-acetylaminofluorene (2-AAF) was used as a diagnostic mutagen while the antimutagenicity of the tea extracts were tested against *Salmonella typhimurium* strain TA 98, a standard tester strain.

The antimutagenicity of the water-soluble extract and the ethyl acetate-soluble fraction of the water-soluble extract of each tea sample was also tested at two concentrations (2.5 $mg.ml^{-1}$ and 12.5 $mg.ml^{-1}$). It was found that the aqueous phases of the unprocessed teas had a stronger antimutagenic activity than the processed teas. The ethyl acetate extracts exhibited a stronger antimutagenicity than the water-soluble extracts. The antimutagenic potential of the four tea extracts was also compared after standardising the polyphenol content (35 mg per 100 mg tea sample). In this case green and unfermented Rooibos tea had very similar antimutagenic potentials and were both more effective than the fermented Rooibos and black teas.

Introduction

Compounds of plant origin have very diverse structures and functions, but polyphenolics constitute an important sub-group. Among plant polyphenolics, flavonoids are of outstanding importance as they are consumed regularly in Western countries in amounts of at least 25 mg per day (Hertog *et al.*, 1993). They are non-toxic, and have been demonstrated to possess various health protective properties including antioxidative, anticarcinogenic, antimutagenic and anti-tumor promoting properties (Edenharder & Tang, 1997).

The Rooibos plant (*Aspalathus linearis)* is a leguminous shrub indigenous to the Cedarberg mountains around Clanwilliam. It's leaves and stems are used for the manufacture of Rooibos tea that is consumed by an ever-increasing number of people around the world. The Rooibos plant is recognised as one of the relatively few economic plants that has made the transformation from a local wild resource to a cultivated crop in the 20th century (Ferreira *et al.*, 1995).

Rooibos tea is rich in volatile components, polyphenols and minerals. The anticarcinogenic and antimutagenic effects of polyphenols suggest that Rooibos tea may be effective in helping prevent mutations that may lead to the development of cancer. Beneficial effects of Rooibos tea may therefore be linked to its polyphenolic composition and associated antioxidant activity (Ferreira *et al.*, 1995).

Much research into the antimutagenic and anticarcinogenic potential of green tea (*Camellia sinensis*) has already been done. Green tea has been the most popular beverage, at least in some parts of the world, since ancient times (Mukhtar *et al.*, 1992), and epidemiological studies have shown that the consumption of green tea lowers the risk for colon cancer (Kato *et al.*, 1990). It has also been reported that epigallocatechin gallate (EGCG), a major polyphenol in green tea, inhibits the promotion of induced cancers in animals (Hayatsu *et al.*, 1992). Experimental studies have shown that green tea affords protection against carcinogenic effects of a number of structurally diverse chemicals, including major classes of chemical carcinogens such as polycyclic aromatic hydrocarbons and nitroso compounds as well as indirect-

acting mutagens. These include aromatic and heterocyclic amines, mycotoxins and polycyclic aromatic hydrocarbons (Bu–Abbas *et al.*, 1997).

Initial studies tended to test only green teas, but recent experimental research has demonstrated cancer inhibition for black types (*Camellia sinensis*) as well (Blot *et al.*, 1997). Polyphenols in the form of catechins compose approximately 30% of the dry weight of the tea leaf (*Camellia sinensis*). This percentage is reduced during the manufacture of black tea from the green tea leaves. Theaflavins and thearubigens are formed during the manufacturing of black tea. Theaflavins are a group of flavonoid biopolymers that exhibit a orange-red colour in solution and are important contributors to the "brightness" and astringency of tea. Thearubigens are undefined, water soluble polyphenolic substances thought to be the brown to black pigments of tea (Balentine, 1997).

The aim of this study was to determine the antimutagenic activity of the water-soluble and ethyl acetate extracts of fermented Rooibos, unfermented Rooibos, green and black teas.

Materials and methods

Preparation of tea extracts

Preparation of water-soluble tea extracts - Water extracts were prepared from 1 kg representative samples of fermented Rooibos, unfermented Rooibos (kindly supplied by Dr E. Joubert, ARC-Infruitec/Nietvoorbij), green and black teas (kindly supplied by Zeno Apostolides, Department of Biochemistry, University of Pretoria) that were pulverised in a laboratory hammermill (Serial no 401 Scientific RSA). One litre of boiling distilled water was added to 100 g of the finely ground tea sample and placed in a boiling waterbath for 30 min. In the case of the green and black tea samples, their water soluble solids are extracted much easier than the Rooibos tea samples and they were therefore only placed in the waterbath for 5 min. The extract was filtered (Whatman No. 1 filter paper), frozen at –18°C and then freeze-dried for 5 days (Dr. C.F. Hansmann, ARC-Infruitec/Nietvoorbij, 1998, personal communication – Atlas commercial freeze-dryer, Copenhagen, Denmark). The soluble solid content

of each tea extract was determined in triplicate gravimetrically after drying in a vacuum oven for 120 min. This was done by evaporating a 20 ml aliquot of tea sample from a pre-weighed moisture dish and then determining the weight of the soluble solids left in the dish.

Preparation of ethyl acetate-soluble tea extracts - A reconstituted water extract solution (20 g per 500 ml) of fermented Rooibos, unfermented Rooibos, green and black tea was prepared and extracted with ethyl acetate (250 ml). The liquid-liquid extraction was repeated 10 times and the ethyl acetate phases were pooled before vacuum evaporation (37°C) of the solvent using a Büchi Rotavapor to obtain an oily residue. A minimal amount (50 ml) of distilled water was added to dissolve the residue and the solution was then frozen before being freeze-dried for five days (Dr. C.F. Hansmann, ARC-Infruitec/Nietvoorbij, 1998, personal communication – Atlas commercial freeze-dryer, Copenhagen, Denmark).

Procedure for determining total polyphenol content and total flavonoids of water extracts

Four dilution series were made up using 0.1 g fermented Rooibos or 0.05 g unfermented Rooibos, green and black tea water extract which were diluted to 100 ml with distilled water. Unfermented Rooibos tea has a much higher soluble solid content than the fermented Rooibos and it was therefore decided to halve the amount of unfermented Rooibos used. Of the fermented Rooibos dilution, 6 and 10 ml was respectively made up to 50 ml and 1 ml of these dilutions was then used in the reaction flasks to determine the total polyphenol and flavonoid content according to the methods of Singleton & Rossi (1965) and Kramling & Singleton (1969). Of the unfermented Rooibos, green and black tea dilutions 2, 4 and 5 ml respectively was made up to 50 ml and 1 ml of these dilutions was then used in the reaction flasks to determine the total polyphenol and flavonoid content using the above-mentioned methods.

The four tea extracts were then prepared on a standard polyphenol basis (35 mg per tea sample). These stock solutions of each tea extract were then diluted five times for use in the Ames test.

The Ames Test

Procedure for preparation of media - Thirty ml of minimal agar (1.5% (m/v) agar and 2.0% (m/v) glucose sterilised separately), was poured into petri-dishes (100 mm x 15 mm) and allowed to set. A 100 ml stock solution of sterile top-agar (0.65% (m/v) agar and 0.5% (m/v) NaCl) was prepared, to which a 10 ml stock solution of 0.5 mM L-histidine HCl and 0.5 mM biotin was added. The top agar was maintained at 48°C in a waterbath until used. The small amount of histidine present in the top agar allows the bacterial cells that require histidine for growth to undergo several cell divisions, which enhances the mutagenic effects of the compounds to be tested (Maron & Ames, 1983).

Induction of rat liver enzymes - For general mutagenesis liver homogenates from rats induced with Aroclor 1254 is recommended (Maron & Ames, 1983). The induction procedure is similar to the method of Czygan *et al.* (1973). Fischer 344 male rats weighing approximately 200 g were used. Aroclor 1254 was diluted in corn oil to a concentration of 200 $mg.ml^{-1}$ and a single injection of 500 $mg.kg^{-1}$ was administered to each rat five days before sacrifice (Maron & Ames, 1983).

Procedure for preparation of liver homogenate S-9 fraction - Four Fischer 344 rats treated with Aroclor 1254, were killed by cervical dislocation and placed on their backs on an autopsy board. Their feet were secured with pins and the fur was swabbed thoroughly with 70% ethanol. The skin was cut and the skin flaps folded back and pinned to the autopsy board to avoid getting fur into the abdominal cavity. The muscle layer was swabbed with ethanol before this layer was cut away with sterile scissors. The livers were excised aseptically from the rats and placed in a pre-weighed beaker containing 1 ml sterile chilled 0.15 M KCl per g of wet liver (rat liver weighs 7 - 10 g). The mass of the liver was determined after which it was washed thoroughly with cold sterile KCl to remove haemoglobin which inhibits activity of P450 (Maron & Ames, 1983). The liver was minced with sterile scissors, transferred into stainless steel beakers and homogenised for 30 sec at 9 000 rpm. The homogenate was filtered through four layers of cheesecloth, the filtrate homogenised again

and centrifuged at 9 000 rpm for 10 min. Aliquots (3 ml) of the supernatant (S-9 fraction) were frozen at –80°C in McCartney bottles (Maron & Ames, 1983).

Preparation of S-9 mix – The prepared S-9 (Maron & Ames, 1983) was thawed at room temperature and kept on ice during use for only one day after which it was discarded. The S-9 mix used in the assay contained the following per 50 ml: 2.0 ml S-9 fraction (Aroclor-1254-induced); 1.0 ml $MgCl_2$-KCl salts; 0.25 ml 1M glucose-6-phosphate; 2.0 ml 0.1M NADP (Boeringer Mannheim Inc.); 25 ml 0.2 M phosphate buffer (pH 7.4); and 19.75 ml sterile distilled water. Stock solutions of 0.1 M NADP and 1 M glucose-6-phosphate were prepared with sterile distilled water and stored at –20°C. The stock salt solutions and phosphate buffer were prepared likewise, autoclaved, and stored in a refrigerator. The S-9 mix was freshly prepared each day and could be kept for several hours on ice. Samples were also tested for bacterial contamination by adding 0.5 ml S-9 to 10 ml top-agar and plating on minimal glucose agar. After 48 hours incubation at 37°C no bacterial growth indicated that the S-9 was not contaminated (Maron & Ames, 1983).

Preparation of mutagen - Two milligrams of 2-acetylaminofluorene (2-AAF) was dissolved in 1 ml di-methyl sulphoxide (DMSO) to produce a stock solution of mutagen. 0.1 ml of the stock solution was then made up to 2 ml using DMSO. From this solution 0.5 ml was again made up to 2 ml to produce a final concentration of 2.5 µg 2-AAF per 0.1 ml DMSO.

Preparation of fermented Rooibos, unfermented Rooibos, green and black tea extracts - A 2.5 g per 100 ml solution of the water or ethyl-acetate tea extract was prepared by adding 2.5 g of freeze-dried tea extract to 100 ml of just boiled (100°C) distilled water. One hundred millilitres was distributed amongst a few centrifuge tubes and centrifuged at 3 - 4 g for 30 min at room temperature. The supernatant was filtered under vacuum through a 0.45 µm acetate, membrane filter (Micron Separations Inc.). This filtrate was then sterilised through a 0.22 µm acetate syringe filter and the filtrate was used to

prepare a dilution series. The tea concentrations prepared were 0.025, 0.0125, 0.0025, 0.00125 and 0.00025 $g.ml^{-1}$.

Procedure for setting up the Plate Incorporation Assay - The bacterial tester strains TA 98 and TA 102 (Maron & Ames, 1983) were incubated overnight at 37°C in an Oxoid Broth No. 2 solution. For the standard pour plate assay, 0.1 ml of the overnight Oxoid broth culture of the bacterial tester strain was added to 2 ml of top agar at 45°C. The mutagen (0.1 ml), tea extract (0.1 ml) and S-9 mix (0.5 ml) were added to the reaction mixture. The contents were mixed immediately by gently blending using a Vortex mixer and poured over the surface of the minimal glucose agar plate. The plates were gently tilted back-and-forth to evenly distribute the top agar layer. The entire operation must be carried out in 20 sec or less once the S-9 mix has been added. It is important to follow these time limits otherwise the top-agar starts to set in mid-operation and an uneven surface results, which makes counting of the revertant colonies difficult. The plates were allowed to harden for a few min, inverted and incubated upside down in the dark at 37°C. The plates must be kept in the dark to avoid the effects of light on photo-sensitive chemicals (Maron & Ames, 1983). In addition to the test plates, control plates of bacteria without tea extract, mutagen and S-9 mix were also prepared.

After two days the histidine revertants (prototrophs) were counted and the presence of a slight background growth of bacteria on the plate confirmed growth due to the small amount of histidine added to the medium. A background lawn that is thin compared to the lawn on the negative control plate is evidence of bacterial toxicity. Colonies that appear on the plate that have no background lawn are not revertants and should therefore not be counted. These colonies arise from the surviving bacteria that grow off the histidine present in the top agar.

The percentage inhibition values can be determined as follows:

$$\% \text{ Inhibition} = 1 - \frac{\text{(No. His}^{+} \text{ revertants in presence of tea}}{\text{No. His}^{+} \text{ revertants in absence of tea)}} \times 100\%$$

Results and discussion

Soluble solids and polyphenolic content of water-soluble extracts

The results obtained showed that green and black tea had a much higher water-soluble solid content than the Rooibos tea extracts (Table 1). Yen & Chen (1996) reported similar results for green and black tea extracts. Fermented Rooibos tea extract had a lower soluble solid content as well as a lower total polyphenolic content than unfermented Rooibos tea extract (Table 1). The green tea extract has a much higher flavonoid content than both the fermented Rooibos and unfermented Rooibos extracts. In contrast, the black tea extract had a lower flavonoid content than the green tea extract, but also had a higher flavonoid content than the fermented Rooibos tea extract.

The reasons for the differences in the soluble solid and flavonoid content may be due to the difference in the extraction times of the teas during the preparation of the water-soluble extracts. The Rooibos samples were extracted for 30 min whereas the green and black teas were only extracted for five min. Another difference is that Rooibos is manufactured from the stems and leaves of the herbal *Aspalathus linearis* plant, whereas green and black tea is manufactured from only the leaves of the hybrid *Camellia sinensis* plant. Despite these differences the Rooibos samples still have the lowest soluble solid content of the four teas and fermented Rooibos has the lowest flavonoid content.

Salmonella typhimurium mutagenicity (Ames) test

The Ames test is a very sensitive bacterial test for detecting chemical mutagens. The compounds are tested on petri dishes with several specially constructed mutants of *Salmonella typhimurium* selected for sensitivity and specificity in being reverted from a histidine requirement back to prototrophy by a wide variety of mutagens (Maron & Ames, 1983).

For this study tester strain TA 98 was selected as it is a primary tester strain recommended for testing general mutagenicity. TA 98 detects various frameshift mutagens. Frameshift mutagens can stabilise the shifted pairing that often occurs in repetitive sequences or 'hot spots' in the DNA, resulting in

Table 1. Water soluble solid and polyphenolic contents of the tea extracts[a].

Tea extract*	Water soluble solids ($g.100ml^{-1}$)	Total polyphenols ($mg.100\ ml^{-1}$)	Total non-flavanoids ($mg.100\ ml^{-1}$)	Total flavanoids ($mg.100\ ml^{-1}$)
Green tea	3.03±0.02	51.67±0.07	0.08±0.03	51.59±0.27
Fermented Rooibos	1.29±0.05	33.46±0.21	7.37±0.07	26.09±0.16
Unfermented Rooibos	1.79±0.03	42.24±0.08	1.51±0.14	40.73±0.20
Black tea	2.33±0.02	36.99±0.68	1.49±0.21	35.50±0.35

[a] Results are the average of triplicates ± SD.

*Extraction time: Rooibos = 30 min, green and black = 5 min

a frameshift mutation which restores the correct reading frame for histidine synthesis (Maron & Ames, 1983).

The Ames test was specially adapted to incorporate the freeze-dried tea extracts. This was done by slightly increasing the concentration of the histidine and biotin solution in the top-agar to counteract the dilution effect caused by adding the reconstituted tea extract. A known mutagen, 2-acetylaminofluorene (2-AAF) was used to test the tea extracts as it is one of the best studied chemical carcinogens. This carcinogen which induces tumours especially in the liver, sebaceous ear duct gland, mammary gland and epithelium of the small intestine in the rat is N-hydroxylated *in vivo,* primarily in the liver, to N-hydroxy-2-acetylaminofluorene. This N-hydroxy metabolite is a much more potent carcinogen than the parent compound at the sites at which 2-AAF is carcinogenic. In addition, it induces tumours at sites of application where 2-AAF has little or no activity (Maron & Ames, 1983).

(i) Water-soluble tea extracts

Water extracts were tested using the Ames test to determine the antimutagenic activity of the soluble solids of the different teas. The different soluble solids found in the different teas influences the antimutagenic potential of the tea. The data depicted in the graphs (Fig. 1 to 4) represent the results of the Ames tests for the four tea types at five different concentrations. As the concentration of the teas was increased the number of revertant colonies decreased in all cases, indicating increased antimutagenic activity. In none of the tests were the tea concentrations toxic to the bacteria. The percentage inhibition values for the various tea types were also an indication that the higher the concentration of tea the stronger the antimutagenic activity (Table 2).

The tea concentrations of 2.5 $mg.ml^{-1}$ and 12.5 $mg.ml^{-1}$ generally showed the smallest standard deviation in all the tea types and were neither the lowest or highest concentrations of tea used in the previous Ames tests (Fig. 1 to 4). It was therefore decided to use these two concentrations to test all the tea extracts simultaneously using the Ames test to indicate which tea sample had the highest antimutagenic potential. Five repeats of each sample

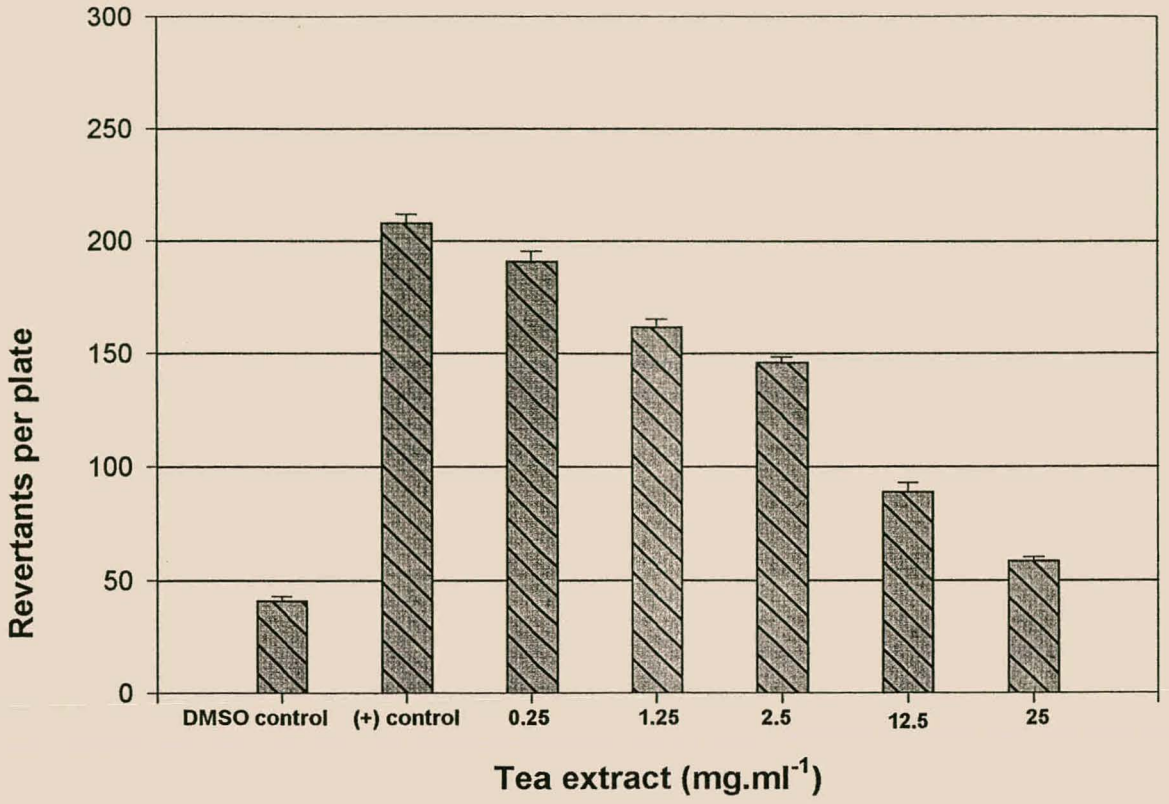

Figure 1. Antimutagenic potential in fermented Rooibos tea. Data are averages of triplicates with error bars representing standard deviation (SD), [Treatments: DMSO control, positive control (2-AAF) and (+) control with different tea concentrations].

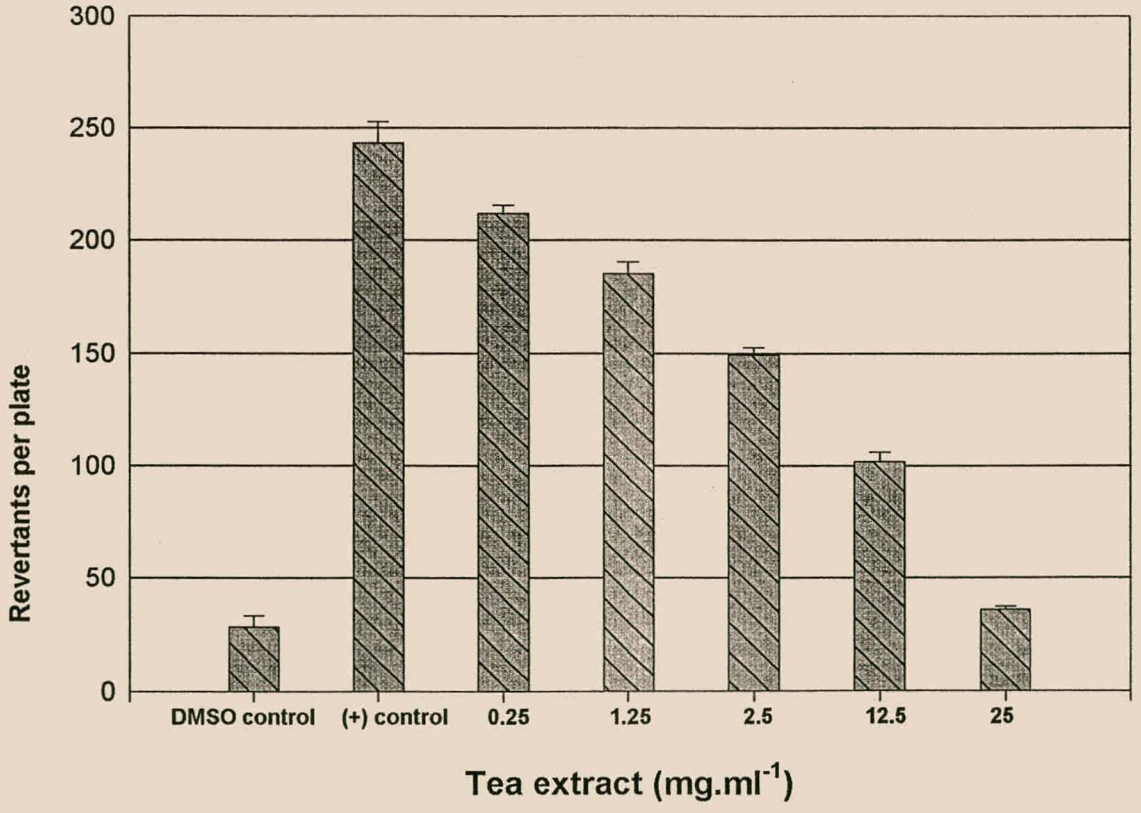

Figure 2. Antimutagenic potential in unfermented Rooibos tea. Data are averages of triplicates with error bars representing standard deviation (SD), [Treatments: DMSO control, positive control (2-AAF) and (+) control with different tea concentrations].

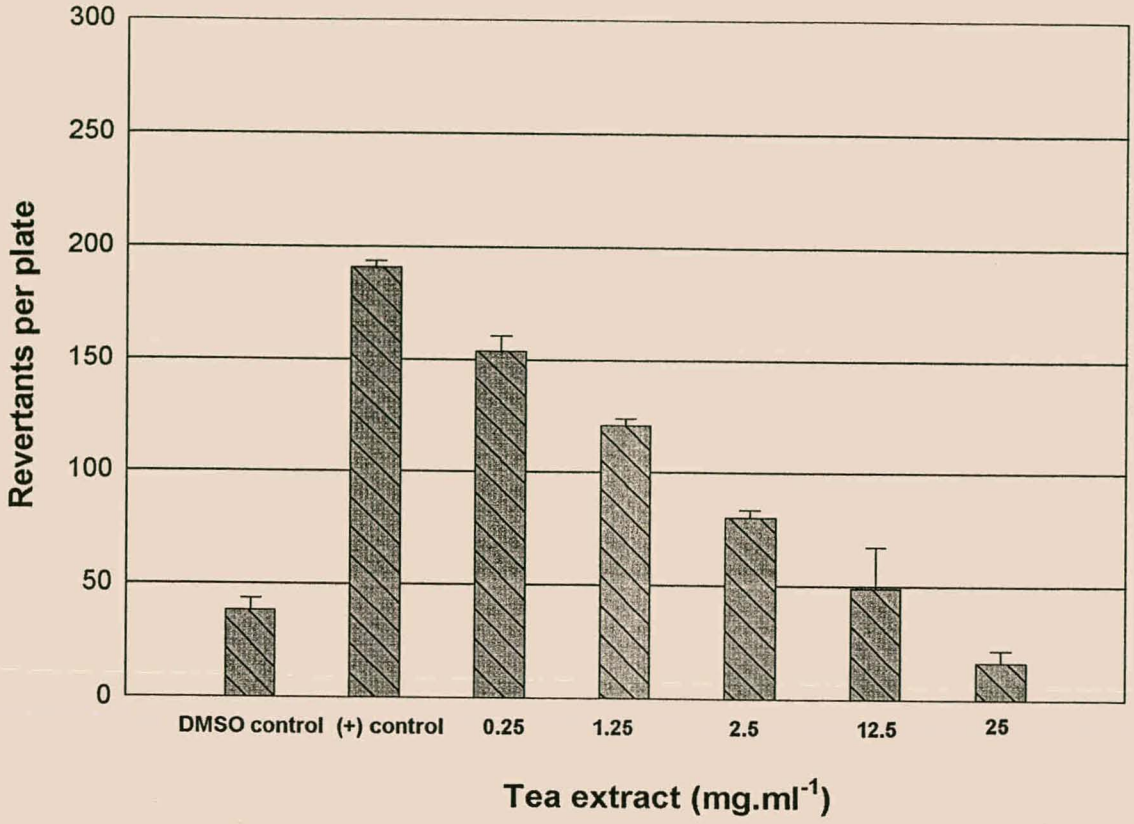

Figure 3. Antimutagenic potential in green tea. Data are averages of triplicates with error bars representing standard deviation (SD), [Treatments: DMSO control, positive control (2-AAF) and (+) control with different tea concentrations].

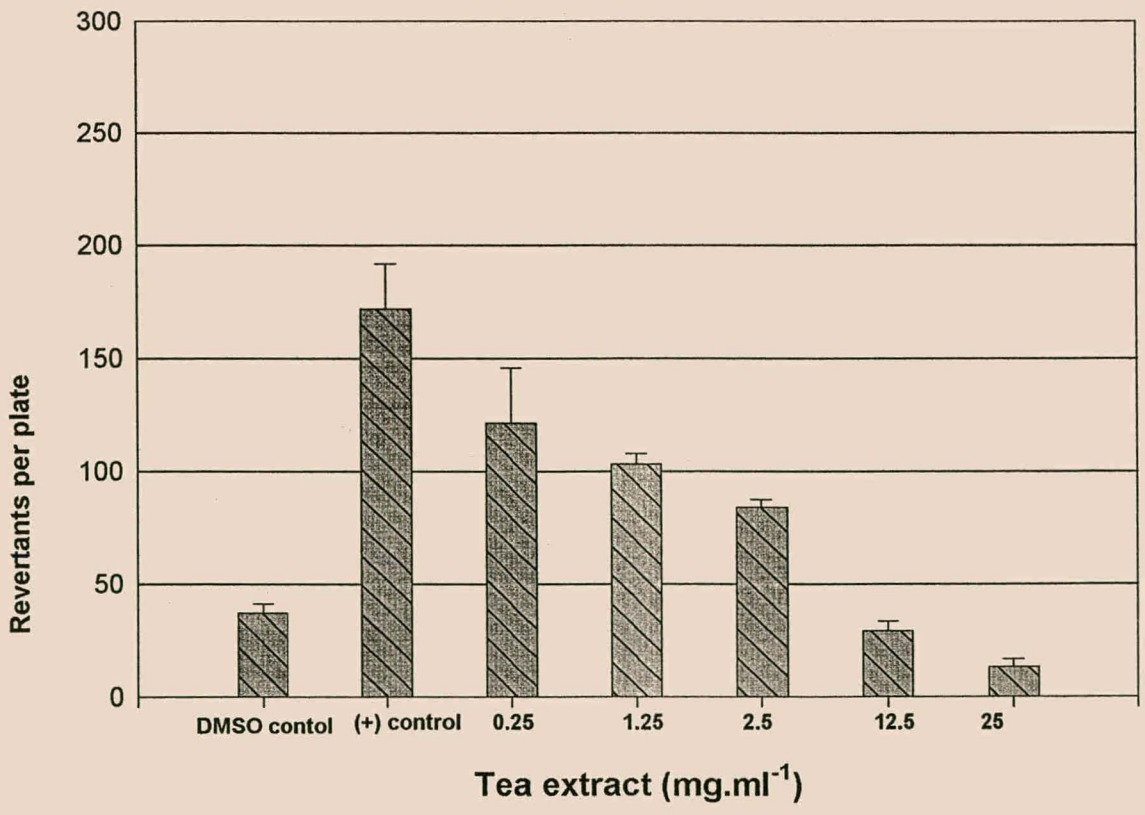

Figure 4. Antimutagenic potential in black tea. Data are averages of triplicates with error bars representing standard deviation (SD), [Treatments: DMSO control, positive control (2-AAF) and (+) control with different tea concentrations].

Table 2. Percentage inhibition values for fermented Rooibos (RB), unfermented Rooibos, green and black tea types tested using the Ames test[a].

Tea extract*	0.25 ($mg.ml^{-1}$)	1.25 ($mg.ml^{-1}$)	2.50 ($mg.ml^{-1}$)	12.5 ($mg.ml^{-1}$)	25.00 ($mg.ml^{-1}$)
Fermented RB	8.1±2.6	22.2±3.3	29.7±3.5	57.2±3.0	71.8±3.8
Unfermented RB	12.9±3.00	23.8±4.5	38.6±2.0	58.1±3.8	85.3±2.5
Green	29.3±2.6	41.0±2.5	51.1±4.3	83.1±8.5	92.4±3.0
Black	19.4±2.4	36.8±7.3	58.2±2.6	74.4±3.3	91.4±2.4

[a]Results are the average of triplicates ± SD.

*Extraction time: Rooibos = 30 min, green and black = 5 min.

were tested and the Ames test was performed twice. The results of the Ames test, using the *Salmonella* strain TA 98 (Fig. 5), showed that green tea had the highest percentage inhibition (Table 3) at both 2.5 $mg.ml^{-1}$ and the 12.5 $mg.ml^{-1}$ concentrations. Unfermented Rooibos tea had the second highest percentage inhibition at both concentrations (2.5 $mg.ml^{-1}$ and 12.5 $mg.ml^{-1}$) which was not that much lower than that of the green tea. Fermented Rooibos tea had a higher percentage inhibition at 2.5 $mg.ml^{-1}$ than the black tea but a very similar percentage inhibition at 12.5 $mg.ml^{-1}$ (Table 3).

When comparing the percentage inhibition values in Table 2 to those in Table 3 it is evident that for all teas of concentration 2.5 $mg.ml^{-1}$ the values in Table 3 are lower than in Table 2. The same applies to teas of concentration 12.5 $mg.ml^{-1}$ except unfermented Rooibos. There is no definite explanation for this although the time elapsed between experiments could have contributed to a spontaneous loss of activity. This phenomenon must be researched further.

(ii) Ethyl acetate extracts

The ethyl acetate extract (water soluble) afforded selective extraction of the flavonoids found in the water soluble extract (Dr. E. Joubert, ARC-Infruitec/Nietvoorbij, 1998, personal communication). Flavonoids such as catechins are generally thought to be responsible for the antimutagenic activity in green tea extracts (Mukhtar *et al.*, 1992). It is thus important to test the ethyl acetate extract separately from the water soluble extract with the Ames test using the four tea extracts.

Ethyl acetate extracts of the four tea types were also tested with the Ames test using both the TA 98 strain (Fig. 6). From the data, it was clear that the green tea ethyl acetate extract exhibited the highest antimutagenic activity followed by unfermented Rooibos. In this series of tests, the fermented Rooibos extract had a very similar antimutagenic activity to that of the black tea. The overall percentage inhibition of each tea extract was however 10 to 25% higher than that found in the test using the water-soluble tea extracts (Tables 3 and 4).

The data (Tables 3 and 4) showed that the green and unfermented Rooibos produced higher inhibition of revertant colonies than the fermented

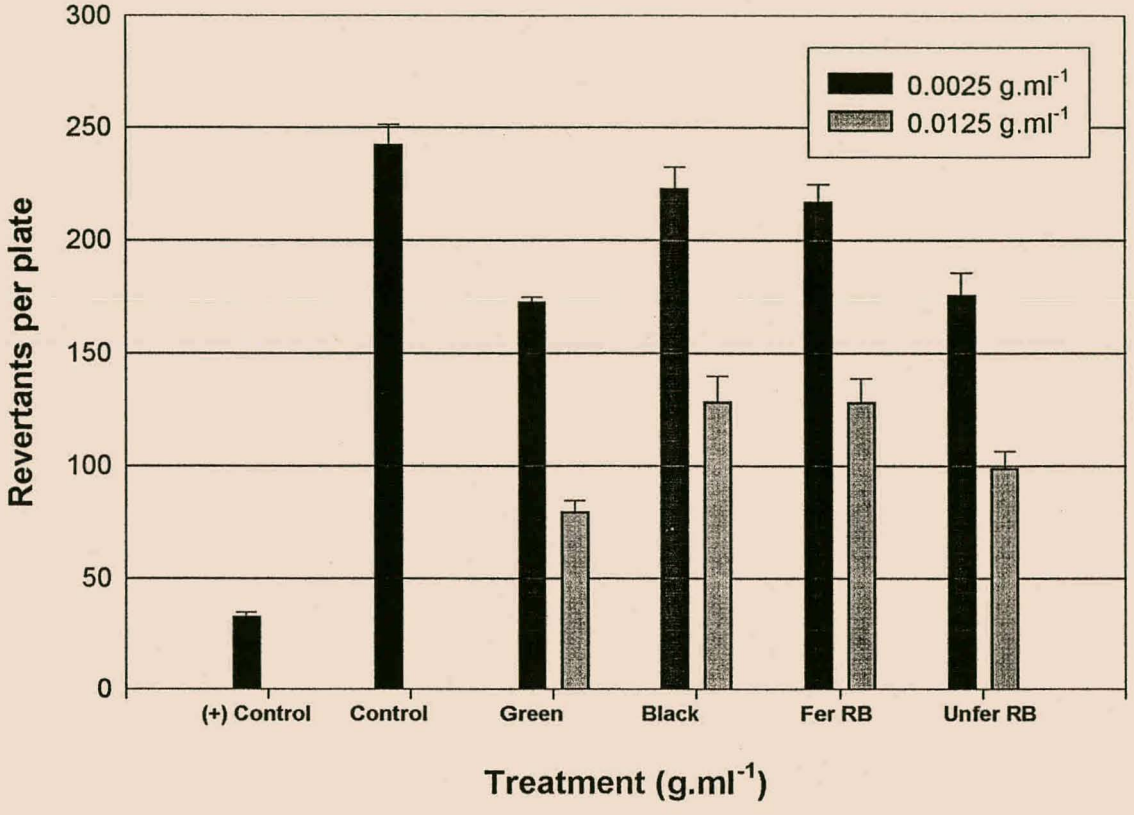

Figure 5. Antimutagenic potential in the water soluble extract of fermented Rooibos (Fer RB), unfermented Rooibos (Unfer RB), green and black tea extracts. Data are averages of triplicates with error bars representing standard deviation [Treatments: DMSO control, (+) control (2-AAF) and (+) control with different tea concentrations].

Table 3. Percentage inhibition results for fermented Rooibos, unfermented Rooibos, green and black tea water soluble extracts tested with the Ames test using *Salmonella typhimurium* strain TA 98 at two different soluble solid concentrations[a].

Tea extract*	2.5 (mg.ml^{-1})	12.5 (mg.ml^{-1})
Fermented RB	9.7±3.5	47.3±4.25
Unfermented RB	28.6±6.0	59.1±3.0
Green	33.7±1.25	67.6±3.0
Black	6.9±4.5	46.5±4.5

[a] Results are the average of triplicates ± SD.

*Extraction time: Rooibos = 30 min, green and black = 5 min

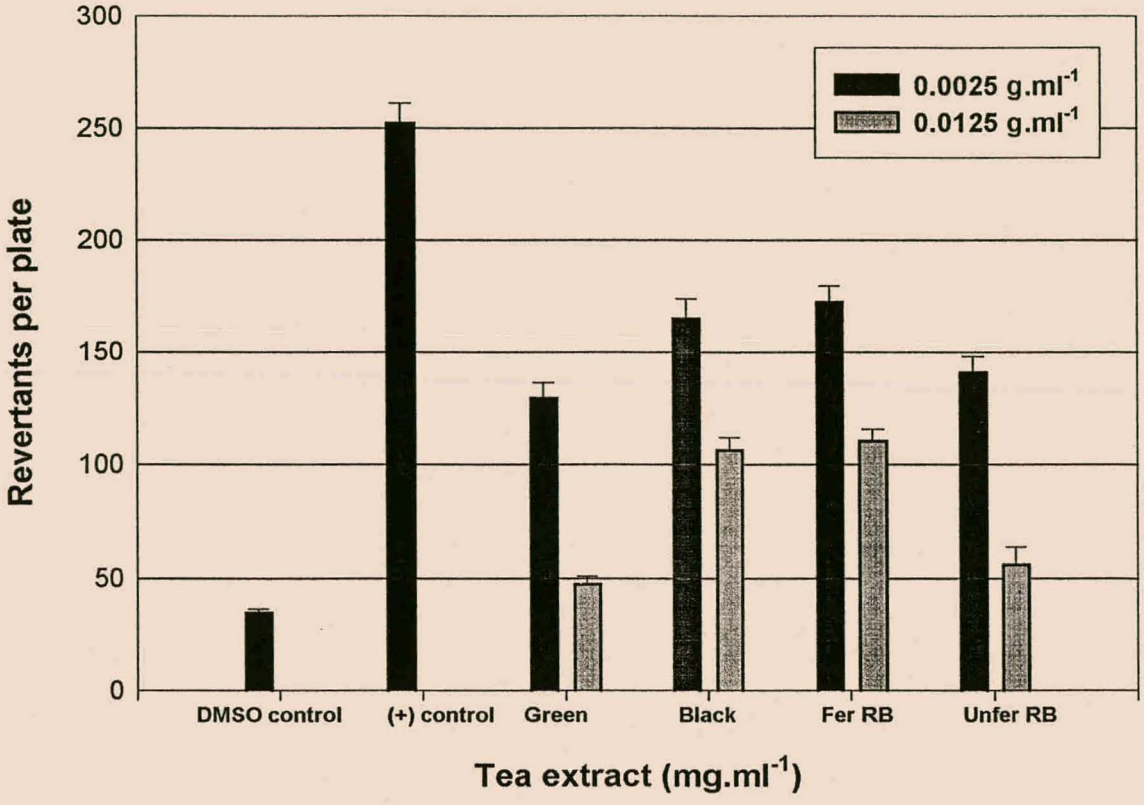

Figure 6. Antimutagenic potential in the ethyl acetate extract of fermented Rooibos (Fer RB), unfermented Rooibos (Unfer RB), green and black tea extracts. Data are averages of triplicates with error bars representing standard deviation [Treatments: DMSO control, (+) control (2-AAF) and (+) control with different tea concentrations].

Table 4. Inhibition results for fermented Rooibos, unfermented Rooibos, green and black tea ethyl acetate extracts tested with the Ames test using *Salmonella typhimurium* strain TA 98 at two soluble solid concentrations[a].

Tea extract*	**2.5 (mg.ml^{-1})**	**12.5 (mg.ml^{-1})**
Fermented RB (%)	33.1±2.5	57.9±3.25
Unfermented RB (%)	44.3±6.0	77.8±4.0
Green (%)	48.8±3.8	81.2±1.3
Black (%)	34.6±4.5	57.9±3.0

[a] Results are the average of triplicates ± SD.

*Extraction time: Rooibos = 30 min, green and black = 5 min

Rooibos and black teas in all tests, therefore exhibiting higher antimutagenic potential. These results therefore show that all the tea types possessed antimutagenic potential although the green and unfermented Rooibos tea were more potent than the fermented Rooibos and black tea. The unfermented Rooibos therefore has a stronger antimutagenic potential than the fermented Rooibos tea. This can probably be explained by the fact that components of the teas are oxidised during the tea processing (Joubert, 1996; Yen *et al.*, 1997). These oxidised products could have either a lower antimutagenic potential than the non-oxidised tea components present in the green tea and unfermented Rooibos or they may have lost their antimutagenic potential.

Green tea was found to contain the highest soluble solid content of the four tea types (Table 1). It is known from the literature that green tea has a very high polyphenol content in the form of catechins (Bu-Abbas *et al.*, 1997) and thus the high antimutagenic activity of the green tea was attributed to this. This may indicate that the polyphenols present in the black tea are not as effective as those found in the unfermented Rooibos tea even though the soluble solid content is higher. A reason for this phenomenon may be due to the fact that the polyphenols found in the unfermented Rooibos and green tea have not been exposed to fermentation (oxidation) (Joubert, 1996, Yen & Chen, 1996) whereas the polyphenols found in the fermented Rooibos and black tea are oxidised (Joubert, 1996, Yen & Chen, 1996).

Standardised polyphenol content

In a separate study the Ames test was performed at a standardised polyphenol content using the fermented Rooibos, unfermented Rooibos, green and black tea extracts. This standardisation is important in determining which of the teas have the most potent antimutagenic activity at a standardised polyphenol level.

In this test the *Salmonella* strain TA 98 was used and the mutagen was again the 2-AAF. At a standard polyphenol content (35 mg per 100 mg tea sample) it was found (Fig. 7) that the difference in the antimutagenic potentials between the tea extracts was less than in the previous Ames tests. At a standard polyphenol content there is therefore not such a large difference

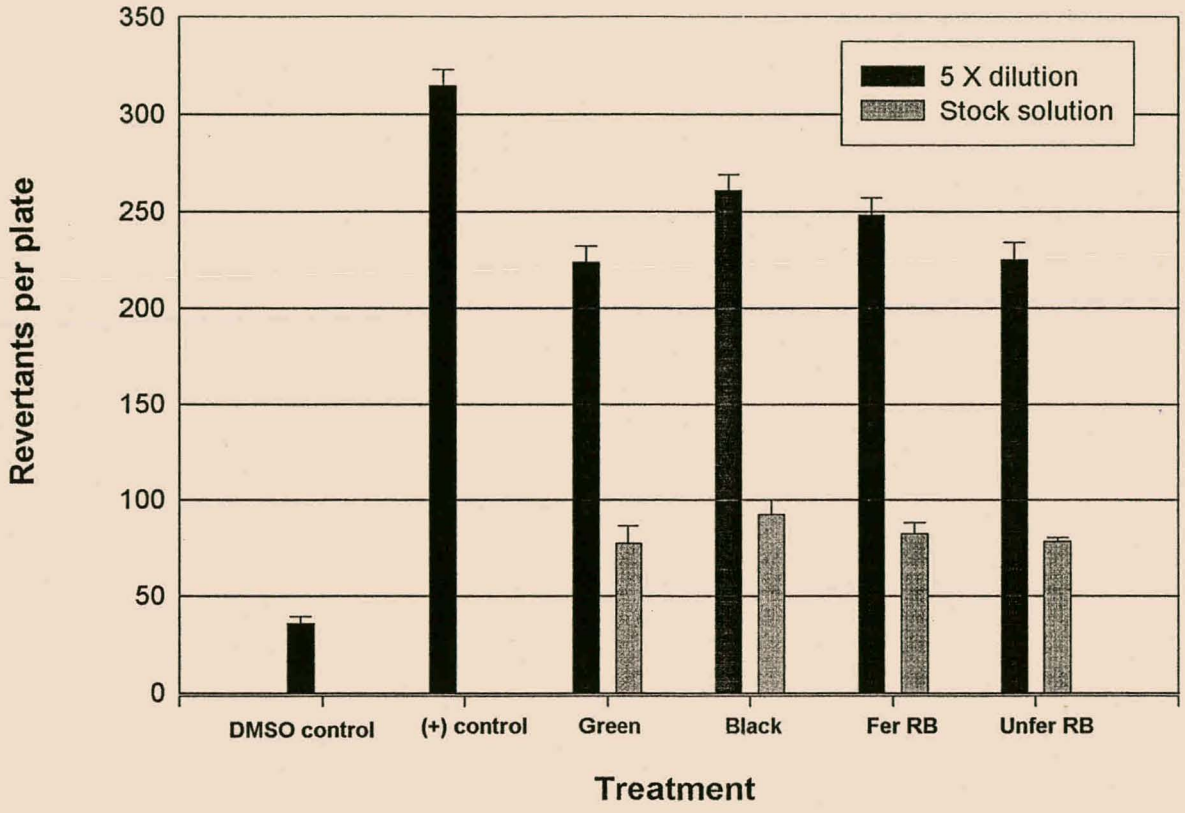

Figure 7. Antimutagenic potential in fermented Rooibos (Fer RB), unfermented Rooibos (Unfer RB), green and black tea extracts at a standard polyphenol content. Data are averages of triplicates with error bars representing standard deviation [Treatments: DMSO control, (+) control (2-AAF) and (+) control with different tea concentrations].

in the antimutagenicity of the four teas. The unfermented Rooibos had a very similar antimutagenic potential to the green tea (Fig. 7). The fermented Rooibos showed a slightly more effective antimutagenic potential than the black tea indicating that its polyphenols after processing were more potent than the black tea's polyphenols. In Table 5 the percentage inhibition values for the four tea types based on a standardised polyphenol level (35 mg per tea sample) are shown, suggesting a higher antimutagenic potential in the fermented Rooibos tea than in the black tea and the very similar potential between the green and unfermented Rooibos tea.

Conclusions

Antimutagenic activity was confirmed in all of the four tea types tested with the Ames test. These included fermented Rooibos, unfermented Rooibos, green and black teas. The results indicated that the unfermented Rooibos and the green tea were more effective than the processed teas, but in all cases the green tea exhibited the highest antimutagenic activity. This suggests that the oxidation step during the processing of the tea can cause a decrease in the antimutagenic activity and the validity of this phenomenon must be further investigated.

Two extracts (water-soluble and ethyl acetate-soluble) of all the tea samples were evaluated. The ethyl acetate tea extracts proved to be more effective than the water soluble extracts in all cases indicating that most of the compounds responsible for antimutagenic potential are probably found in this fraction.

On comparison of the four tea types based on soluble solid content, the green tea showed the most antimutagenic potential and the fermented Rooibos had the weakest antimutagenic potential. When tested at a standard polyphenol level the results showed that the unfermented Rooibos tea had a very similar antimutagenic potential to that of the green tea and both were more effective than the fermented Rooibos and black teas. The fermented Rooibos and black teas had very similar antimutagenic potentials. Green tea can therefore be regarded as having the most potent antimutagenic potential

Table 5. Percentage inhibition results for fermented Rooibos, unfermented Rooibos, green and black tea water soluble extracts tested in the Ames at a standardised polyphenol content (35 mg per tea sample) using *Salmonella typhimurium* TA 98[a].

Tea extract*	**Stock solution**	**5X dilution**
Fermented RB	20.3±3.7	73.0 ±2.5
Unfermented RB	28.5 ±3.5	75.1 ±2.5
Green	29.0 ±2.5	75.5 ±3.7
Black	17.2 ±4.1	70.5± 3.0

[a]Results are the average of triplicates ± SD.

*Extraction time: Rooibos = 30 min, green and black = 5 min

on an equal soluble solid basis with unfermented Rooibos being the next most effective tea.

These results suggest that unfermented Rooibos tea, together with the much researched green tea, may have chemo-preventive properties as its antimutagenic potential is evident in both the water-soluble and ethyl acetate-soluble fractions. The processed teas have a very similar antimutagenic potential although at a standardised polyphenol level it was proven that fermented Rooibos was more effective than the black tea.

Rooibos tea has already been proved to have many properties beneficial to human health as summarised by Joubert & Ferreira (1996). Rooibos tea has now also been proven to have antimutagenic properties which may lead to the prevention of certain types of cancer (Bronzetti, 1994). This, together with the other properties associated with Rooibos tea can only help promote the use of Rooibos as a healthy tea.

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CHAPTER 4

CHANGES IN THE ANTIMUTAGENIC POTENTIAL OF ROOIBOS TEA DURING THE PROCESSING STAGES

Abstract

The antimutagenic potential of Rooibos tea samples from the five major processing stages was tested with the Ames test. These included: unfermented tea, fermented tea, fermented sun-dried tea, tea before steam pasteurisation and tea after steam pasteurisation. Ten random samples were collected from each processing stage. From these samples aqueous phase extracts were prepared and tested at two soluble solid concentrations (2.5 $mg.ml^{-1}$ and 12.5 $mg.ml^{-1}$). The *Salmonella typhimurium* strain TA 98 was used in all the Ames tests and the mutagen was 2-acetylaminofluorene. Results indicated that the fermented tea had a lower antimutagenic potential than the green unfermented tea. It was found that it was mainly during fermentation that the significant drop in the antimutagenic potential of the tea occurred. The remaining processing stages, sun-drying and steam pasteurisation also caused decreases in the antimutagenic potential of the tea but not to the same extent as found during fermentation. The antimutagenic potential of store-purchased Rooibos tea samples from five different retailers was also tested. The tea was tested at two concentrations (12.5 and 2.5 $mg.ml^{-1}$) and the results for both concentrations in all the teas indicated that processing of the tea results in tea with a more-or-less uniform antimutagenic potential. The antimutagenic potential of a crude extract containing 40% aspalathin, a flavonoid unique to Rooibos tea, was also tested in the Ames test. The results confirmed that the crude aspalathin extract exhibited antimutagenic potential.

Introduction

The Rooibos plant (*Aspalathus linearis*) grows in the mountains around Clanwilliam in its natural state in an area of winter rainfall and coarse sandy

soil (Morton, 1983). Seeds are planted in the late summer and the Rooibos plants are cultivated for approximately 18 months before the first harvesting. The harvest, consisting of leaves and fine stems (Ferreira *et al.*, 1995), is cut into small pieces (4.5 mm), placed in heaps in the sunlight, sprayed with water and then bruised between rollers to trigger the fermentation process which lasts approximately 12 h. After fermentation, the Rooibos is spread out thinly in the hot sun for ca. 12 h to dry, and then sucked up by special vacuum pumps. After sieving to remove coarse material, consisting mainly of stems, the tea is sterilised by steam pasteurisation, finely weighed, packed and marketed under various brand names, either in tea bags or in loose leaf form (Dr. E. Joubert, 1998, ARC-Infruitec Nietvoorbij, personal communication).

The flavonoid composition of Rooibos tea is unique as it contains aspalathin (Koeppen & Roux, 1966), which has only been reported in Rooibos tea and another rare compound nothofagin (Hillis & Inoue, 1967), which was previously only isolated from *Nothofagus fusca* (red beech) (Joubert & Ferreira, 1996). Aspalathin and nothofagin constitute approximately 0.55% and 0.9%, respectively, of the soluble solid content of processed Rooibos tea (Joubert, 1994; Ferreira *et al.*, 1995). The flavonoid fraction of Rooibos tea also includes the flavones orientin, iso-orientin, vitexin, iso-vitexin, chrysoeriol, 5,7,4'-trihydroxy-3-methoxy flavone and luteolin and the flavanols iso-quercitrin and rutin, and their aglycone, quercetin (Joubert & Ferreira, 1996; Rabe *et al.*, 1994) as well as the flavanol (+)-catechin (Joubert, 1996).

It has been reported that during processing the aspalathin and nothofagin content decreased, especially during the fermentation step (Joubert, 1996). Processing is also believed to result in the formation of other flavonoids, such as flavanones, dihydro-2,3-orientin and dihydro-3,4-iso-orientin that are formed from aspalathin (Joubert, 1996). Fermented Rooibos tea contains approximately 7% of the dihydrochalcones originally present in the unfermented Rooibos tea (Joubert, 1996). The ratio of dihydrochalcones to total ethyl acetate soluble polyphenols also decreases with processing. The decrease in dihydrochalcones has been attributed to enzymatic and chemical oxidation of polyphenols. The nature of these reactions is still unknown but enzymes such as polyphenol oxidase and peroxidase could play a role (Joubert, 1996). It has also been reported that the polyphenolic content

(Chapter 3 of this thesis) and composition of the unfermented (unprocessed), Rooibos tea differs to that of the fermented (processed) Rooibos tea (Joubert, 1996). In Chapter 3 it was found that the antimutagenic potential of the unfermented Rooibos tea was much more effective than that of the fermented Rooibos tea. It was thus postulated that the oxidation of compounds in the tea during fermentation may be the cause for this decrease in antimutagenic potential.

The aim of this study was to determine the antimutagenic potential during the five stages of Rooibos tea processing using the Ames test. The samples included unfermented (unprocessed) tea, tea after fermentation, tea after sun-drying, tea before steam pasteurisation and tea after steam pasteurisation. The antimutagenic potential of store-purchased Rooibos tea was also investigated as well as the antimutagenic potential of a crude extract containing ca. 40% aspalathin kindly provided by Dr. E Joubert, ARC-Infruitec/Nietvoorbij.

Materials and methods

Preparation of tea samples

Collection of tea samples - Ten random samples (Table 1) from the five major Rooibos processing stages were collected from Rooibos Ltd. in Clanwilliam during the 1998 season. The samples weighed about 1 kg each. Unfermented and fermented tea, and sun-dried fermented tea were collected at the processing yard. Samples were also collected before and after steam pasteurisation. The unfermented Rooibos tea and the wet, fermented Rooibos tea were then dried in a drying tunnel at 40°C for 48 and 24 h, respectively, in the laboratory and thoroughly mixed. Random 500 g Rooibos tea samples were also purchased from five commercial retailers in the Western Cape.

Preparation of water-soluble tea extracts - Water extracts were prepared from the Rooibos tea samples collected in Clanwilliam and the Rooibos tea purchased from five retailers in Cape Town and Stellenbosch. The tea was

Table 1. Tea samples collected in Clanwilliam.

Processing stage	Size of sample (kg)	No. of samples taken	No. of tests
Unfermented tea, uncut	1	10	30
Fermented, tea (tunnel dried)	1	10	30
Fermented, sun-dried tea	1	10	30
Tea before steam pasteurisation	1	10	30
Tea after steam pasteurisation	1	10	30
Total			150 tests

pulverised in a laboratory hammermill (Serial no 401 Scientific RSA). One litre of boiling distilled water was added to 100 g of each of the finely ground tea samples and placed in a boiling waterbath for 30 min. The extract was filtered (Whatman No. 1 filter paper), frozen at –18°C and then freeze-dried for five days (Dr. C. Hansmann, 1998, ARC-Infruitec/Nietvoorbij, personal communication – Atlas freeze-dryer, Copenhagen, Denmark). The soluble solid content of each tea extract was determined gravimetrically after drying in a vacuum oven for 120 min. This was done by evaporating a 20 ml aliquot tea sample from a pre-weighed moisture dish and then weighing the dish to determine the weight of the soluble solids left in the dish.

Preparation of aspalathin-rich samples

A crude fraction containing 40% aspalathin (kindly supplied to Dr. Joubert by Dr D. Ferreira, Department of Chemistry, University of the Orange Free State, Bloemfontein) was used to prepare a stock solution of 12.5 mg in 1 ml DMSO. This solution was then diluted to give final concentrations of 1.25 $mg.ml^{-1}$ and 6.25×10^{-1} $mg.ml^{-1}$, respectively. This was done to produce concentrations of aspalathin similar to those found in Rooibos tea (Dr. E. Joubert, 1998, ARC-Infruitec/Nietvoorbij, personal communication).

The Ames test

Procedure for preparation of media - Thirty ml of sterile minimal agar (1.5% (m/v) agar and 2.0% (m/v) glucose), was poured into petri-dishes (100 mm x 15 mm) and allowed to set. A 100 ml stock solution of sterile top-agar (0.65% (m/v) agar and 0.5% (m/v) NaCl) was prepared, to which a 10 ml stock solution of 0.5 mM L-histidine HCl and 0.5 mM biotin was added. The agar was maintained at 48°C in a waterbath until used. The small amount of histidine present in the top agar allows the bacterial cells to undergo several cell divisions, which enhances the mutagenic effects of the compounds to be tested (Maron & Ames, 1983).

Induction of rat liver enzymes - For general mutagenesis liver homogenates

from rats induced with Aroclor 1254, is recommended (Maron & Ames, 1983). The induction procedure is similar to the method of Czygan *et al* (1973). Fischer 344 male rats, weighing approximately 200 g, were used. Aroclor 1254 was diluted in corn oil to a concentration of 200 $mg.ml^{-1}$ and a single injection of 500 $mg.kg^{-1}$ was administered to each rat five days before sacrifice (Maron & Ames, 1983).

Procedure for preparation of liver homogenate S-9 fraction - Four Fischer 344 rats induced with Aroclor 1254 were killed by cervical dislocation and placed on their backs on an autopsy board. Their feet were secured with pins and the fur was swabbed thoroughly with 70% ethanol. The skin was cut and the skin flaps folded back and pinned to the autopsy board to avoid getting fur into the abdominal cavity. The muscle layer was swabbed with ethanol before this layer was cut away with sterile scissors. The livers were excised aseptically from the rats and placed in a pre-weighed beaker containing 1 ml sterile chilled 0.15 M KCl per g of wet liver (rat liver weighs 7 - 10 g). The mass of the liver was determined after which it was washed thoroughly with cold sterile KCl to remove haemoglobin which inhibits activity of P450 (Maron & Ames, 1983). The liver was minced with sterile scissors, transferred into stainless steel beakers and homogenised for 30 sec at 9 000 rpm. The homogenate was filtered through four layers of cheesecloth, the filtrate homogenised again and centrifuged at 9 000 rpm for 10 min. Aliquots (3 ml) of the supernatant were frozen at –80°C in McCartney bottles (Maron & Ames, 1983).

Preparation of S-9 mix - A tube of S-9 (Maron & Ames, 1983) was thawed at room temperature and kept on ice during use for only one day after which the remainder was discarded. The S-9 mix used in the assay contained per 50 ml: 2.0 ml S-9 fraction (Aroclor-1254-induced); 1.0 ml $MgCl_2$-KCl salts; 0.25 ml 1M glucose-6-phosphate; 2.0 ml 0.1M NADP (Boeringer Mannheim Inc.); 25 ml 0.2 M phosphate buffer (pH 7.4), and 19.75 ml sterile distilled water. Stock solutions of 0.1 M NADP and 1 M glucose-6-phosphate were prepared with sterile distilled water and stored at –20°C. The stock salt solutions and phosphate buffer were prepared likewise, autoclaved, and stored in a refrigerator. The S-9 mix was freshly prepared each day and could be kept

for several hours on ice. Samples were also tested for bacterial contamination by adding 0.5 ml S-9 to 10 ml top-agar and plating on minimal glucose agar. After 48 hours incubation at 37°C no bacterial growth indicated that the S-9 was not contaminated (Maron & Ames, 1983). Although it is possible to remove most contaminants by filtration of the S-9 mix through a 0.45 μm filter, this method may risk enzyme loss, particularly if there is foaming (Maron & Ames, 1983).

Preparation of mutagen - Two milligrams of 2-acetylaminofluorene (2-AAF) was added to 1 ml di-methyl sulphoxide (DMSO) to produce a stock solution of mutagen. 0.1 ml of the stock solution was then made up to 2 ml using DMSO. From this solution 0.5 ml was again made up to 2 ml to produce a final concentration of 2.5 μg 2-AAF per 0.1 ml DMSO.

Procedure for setting up the Plate Incorporation Assay - The bacterial tester strains *Salmonella typhimurium* TA 98 and TA 102 (Maron & Ames, 1983) were incubated overnight at 37°C in an Oxoid Broth No. 2 solution. For the standard pour plate assay, 0.1 ml of the overnight Oxoid broth culture of the bacterial tester strain was added to 2 ml of top agar at 45°C. The mutagen (0.1 ml), tea extract (0.1 ml) and S-9 mix (0.5 ml) were added to the reaction mixture. The contents were mixed immediately by gently blending using a Vortex mixer and poured over the surface of the minimal glucose agar plate. The plates were tilted back-and-forth gently to evenly distribute the top agar layer. The entire operation must be carried out in 20 sec or less once the S-9 mix has been added. It is important to follow these time limits, since when the top-agar starts to harden in mid-operation an uneven surface results which makes counting of the revertant colonies difficult. The plates were allowed to harden in the dark for a few min before being incubated upside down in the dark at 37°C. The plates must be kept in the dark to avoid the effects of light on photo-sensitive chemicals (Maron & Ames, 1983).

After two days the histidine revertants (prototrophs) were counted and the presence of a slight background growth of bacteria on the plate confirmed growth due to the small amount of histidine added to the medium. In addition to the test plates, control plates of bacteria without tea extract, mutagen and

S-9 mix were also prepared. A background lawn that is thin compared to the lawn on the negative control plate is evidence of bacterial toxicity. Colonies that appear on the plate that have no background lawn are not revertants and should therefore not be counted. These colonies arise from the surviving bacteria that live off the histidine present in the top agar.

The percentage inhibition values can be determined as follows:

$$\% \text{ Inhibition} = 1 - \frac{(\text{No. His}^+ \text{ revertants in presence of tea})}{(\text{No. His}^+ \text{ revertants in absence of tea})} \times 100\%$$

Statistical analyses

Statistical analyses were done on the 300 sets of Ames results for the Rooibos tea from the five processing stages (Table 1). The data was analysed on a completely randomised ANOVA design using the seven treatments ((+) control, control, and five processing stages) and 10 repetitions (Mrs A. Sadie, 1999, Biometrics division, ARC-Infruitec/Nietvoorbij, personal communication). The Students t-test was also performed on the data to identify the significantly different groups within the samples from the seven treatments (Mrs A. Sadie, 1999, Biometrics division, ARC-Infruitec/Nietvoorbij, personal communication).

Results and discussion

Ames test of tea samples using Salmonella typhimurium TA 98

Water-soluble extracts prepared from ten samples from each processing stage were tested in triplicate at two different soluble solid concentrations (12.5 and 2.5 $mg.ml^{-1}$) in the Ames test. These concentrations were chosen to correlate with the concentrations used in Chapter 3 of this thesis. These processing stages included samples of unfermented tea, fermented tea, fermented sun-dried tea, tea not steam pasteurised and steam pasteurised tea. One sample from each stage was tested in triplicate at the same time.

In all ten test sets, the results showed that the unfermented Rooibos tea had a stronger antimutagenic potential than the tea samples from all the

other four remaining stages. An example of the data is given in Fig. 1. The most noticeable drop in antimutagenic potential occurred during the fermentation process, as after this stage, the tea had a much lower antimutagenic potential (8 – 20%) at both concentrations. The higher tea concentration (12.5 $mg.ml^{-1}$) produced higher antimutagenic potentials than the lower concentration (2.5 $mg.ml^{-1}$) in all the processing stages of the tea. In Chapter 3 the higher concentration (12.5 $mg.ml^{-1}$) also proved to have a more effective antimutagenic potential than the lower concentration used (2.5 $mg.ml^{-1}$). The change in antimutagenic potential does not differ as significantly in the remaining four stages as it did after fermentation although the number or revertant colonies did increase indicating a relatively steady decrease in antimutagenic potential as a result of further processing (Fig. 1). The tea from the final stage of processing (after steam pasteurisation) had the lowest antimutagenic potential of all the five stages although it did have significant antimutagenic potential when compared to the control. The inhibition values for the two concentrations of tea used (Tables 2 and 3) confirm that the unfermented Rooibos tea had the strongest antimutagenic potential while the Rooibos collected after steam pasteurisation had the lowest antimutagenic potential.

The data obtained during the Ames testing was statistically analysed on a completely randomised design using the seven treatments ((+) control, control and five processing stages) and 10 repetitions in triplicate. The analysis of variants on data to test for differences between treatments (ANOVA test) was applied (Mrs A. Sadie, 1999, Biometrics division, ARC-Infruitec/Nietvoorbij, personal communication). This test relies on the H_o hypothesis (Mrs A. Sadie, 1999, Biometrics division, ARC-Infruitec/Nietvoorbij, personal communication) which states that all treatments are equal and that no differences are present between treatment means (Snedecor & Cochran, 1980). The resulting Pr>F value is an indication of whether this theory is valid or not. The Pr>F value was 0.0001 for the tea samples of concentration 2.5 $mg.ml^{-1}$ and 0.0001 for the tea samples of concentration 12.5 $mg.ml^{-1}$. These values indicated that the H_o theory was not probable and that the differences in the antimutagenic potentials of the tea samples was in fact due to the different treatments (processing stages).

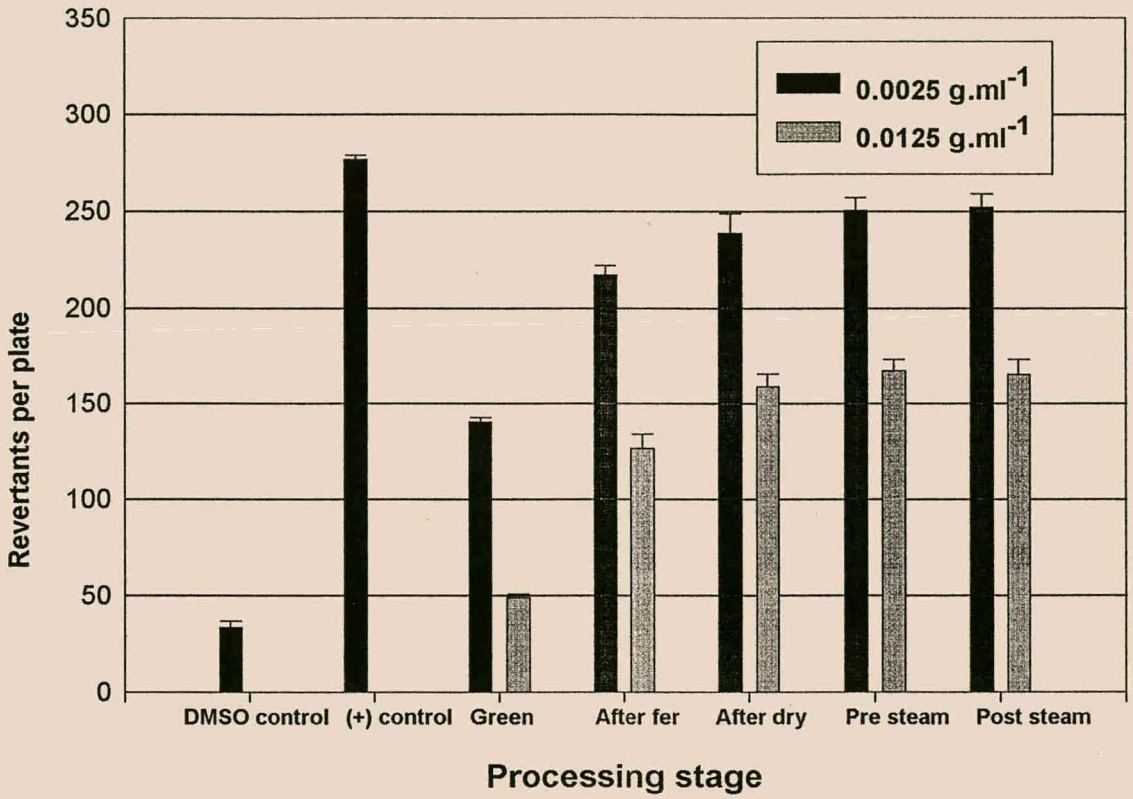

Figure 1. Antimutagenic potential in Rooibos tea from five processing stages (sample 1). Data are averages of triplicates with error bars representing SD [Treatments: DMSO control, (+) control (2-AAF) and (+) control with five different Rooibos tea extracts].

Table 2. Averaged percentage inhibition values[a] for Rooibos (RB) tea samples at soluble solid concentration of 0.0025g.ml^{-1} from the five processing stages tested with the Ames test.

Samples in triplicate	Green RB	After fermentation	After drying	Before steaming	After steaming
1	49.0	21.5	13.7	9.4	8.8
2	29.7	9.7	8.4	4.4	3.0
3	39.3	12.7	8.1	5.1	3.0
4	42.7	24.2	14.7	8.6	9.3
5	39.8	16.0	9.2	9.3	6.0
6	39.8	19.9	13.3	10.5	8.4
7	39.0	12.4	7.9	5.6	3.8
8	29.4	9.4	7.8	3.8	2.4
9	28.4	8.5	6.3	3.5	2.6
10	40.3	12.0	8.3	5.9	3.9

[a]% Inhibition = 1 – (No. His$^+$ revertants in presence tea / No. His$^+$ revertants in absence tea) x 100%

Table 3. Averaged percentage inhibition values[a] for Rooibos (RB) tea samples at soluble solid concentration of 0.0125g.ml^{-1} from the five processing stages tested with the Ames test.

Samples in triplicate	Green RB	After fermentation	After drying	Before steaming	After steaming
1	82.4	54.3	42.6	39.6	40.3
2	77.3	50.3	43.5	37.6	35.4
3	78.9	53.3	48.4	46.6	43.1
4	78.7	52.6	51.9	42.7	39.5
5	76.3	56.2	52.8	50.1	46.5
6	74.3	51.9	48.4	40.7	38.7
7	77.7	52.2	48.5	46.0	42.5
8	75.7	49.3	42.4	32.7	35.0
9	74.9	48.8	43.4	36.7	33.7
10	79.4	53.4	48.7	47.1	44.3

[a]% Inhibition = 1 – (No. His$^+$ revertants in presence tea / No. His$^+$ revertants in absence tea) x 100%

As a result of the ANOVA test indicating that the H_o hypothesis was more than likely not valid, the Students t-test was performed on the data to distinguish which treatments resulted in tea with significantly different antimutagenic potentials (Tables 4 and 5). In this test the significantly different groups were identified. The tea with concentration 2.5 $mg.ml^{-1}$ could be divided up into three basic statistic groups. The tea from the final stage (after steam pasteurisation) was not significantly different from the control without tea (Table 4), as was the case when tea of concentration 12.5 $mg.ml^{-1}$ was used (Table 5). It was during fermentation that the most significant drop in antimutagenic potential occurred.

The tea with the higher soluble solids concentration of 12.5 $mg.ml^{-1}$ could in contrast be divided into four basic statistic groups. The most significant drop in antimutagenic potential occurred again after the fermentation stage. The tea samples from the remaining stages of processing also exhibited antimutagenic potential as the tea from the final stage differs markedly from the positive control. The tea of concentration 12.5 $mg.ml^{-1}$ therefore exhibited a more potent antimutagenic potential than the tea of concentration 2.5 $mg.ml^{-1}$.

The data in both Tables 4 and 5 indicated that the unfermented Rooibos tea had a significantly stronger antimutagenic potential than the fermented Rooibos tea. Fermentation must therefore cause a major decrease in antimutagenic potential of Rooibos tea.

Rooibos tea samples from five retailers

The antimutagenic potential of commercially available Rooibos tea samples was also investigated to determine if the manufacturing process is producing tea of consistent antimutagenic potential. All five samples used from various retailers in the Cape showed similar antimutagenic potentials at the soluble solid concentrations used (12.5 and 2.5 $g.ml^{-1}$)(Fig. 2). These similar antimutagenic potentials can possibly be considered an indication that the processing process applied during the manufacture of Rooibos tea is very consistent and that all Rooibos tea produced and sold has a similar antimutagenic potential.

Table 4. Results of the Pairwise Student's t-Test performed on the Ames data from the five processing stages of Rooibos tea (RB) at soluble solid concentration of 2.5 $mg.ml^{-1}$.

T Grouping		Mean	No. of samples tested in triplicate	Sample
	A	268.55	10	Control
	A	259.72	10	After steaming
B	A	249.43	10	Before steaming
B	A	241.89	10	After drying
B		229.04	10	After fermentation
	C	166.65	10	Unfermented RB
	D	31.72	10	(+) Control

Alpha = 0.05
df = 63
MSE = 916.9787
Critical Value of T = 2.00
Least Significant Difference = 27.062
Means with the same letter are not significantly different
(+) Control: No 2-AAF, S-9 mix or tea extract
Control: no tea extract but DMSO

Table 5. Results of Pairwise Student's t-Test performed of the Ames data from the five processing stages of Rooibos tea (RB) at soluble solid concentration of 12.5 mg.ml^{-1}.

T Grouping		Mean	No. of samples tested in triplicate	Sample
	A	268.55	10	Control
	B	160.78	10	After steaming
	B	154.42	10	Before steaming
C	B	141.99	10	After drying
C		127.43	10	After fermentation
	D	61.28	10	Unfermented RB
	E	31.72	10	(+) Control

Alpha = 0.05
df = 63
MSE = 502.724
Critical Value of T = 2.00
Least Significant Difference = 20.038
Means with the same letter are not significantly different
(+) Control: no 2-AAF, S-9 mix or tea extract
Control: no tea extract but DMSO

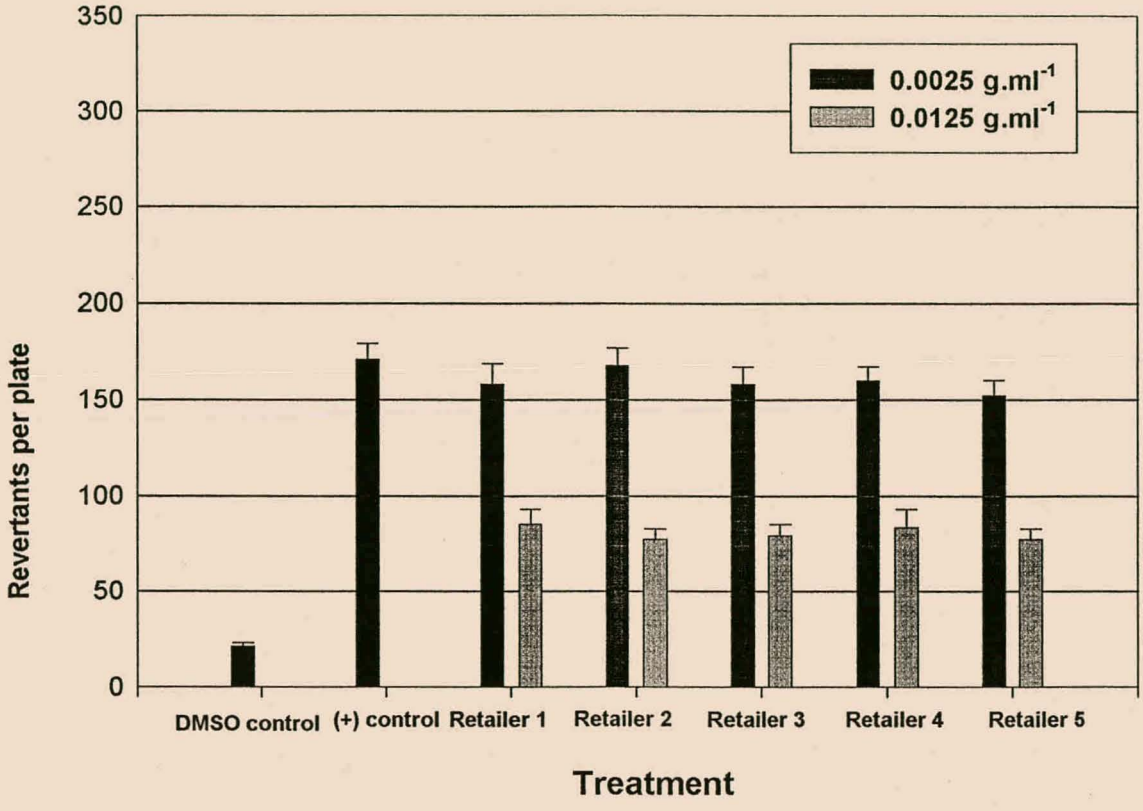

Figure 2. Antimutagenic potential in Rooibos tea from five retailers. Data are averages of triplicates with error bars representing SD [Treatments: DMSO control, (+) control (2-AAF) and (+) control with five different Rooibos tea extracts].

The percentage inhibition values in Table 6 show again how similar the results were, especially at the higher soluble solid concentration (12.5 $mg.ml^{-1}$). The higher concentration (12.5 $mg.ml^{-1}$) of tea used in the study showed much lower colony counts indicating a stronger antimutagenic potential than the lower concentration (2.5 $mg.ml^{-1}$) of tea. This correlates with the data obtained from the statistical analysis of the data from the processing stages as well as the data in Chapter 3.

40% Aspalathin crude samples

The crude aspalathin-rich sample was tested using the Ames test to determine if such a sample contributes to the antimutagenic potential of Rooibos tea. The results were an indication that the crude sample containing 40% aspalathin did exhibit antimutagenic potential (Fig. 3). This was more evident at the higher concentration (1.25 $mg.ml^{-1}$) where the percentage inhibition was 50.13%, than at the lower concentration (6.25×10^{-1} $mg.ml^{-1}$) where the percentage inhibition value was 15.76%. The standard deviations of the results were relatively high compared to the results of previous Ames tests.

Conclusions

The results from the Ames test when testing Rooibos tea from the different processing stages indicated that the fermented Rooibos tea does not have an antimutagenic potential as effective as the unfermented Rooibos tea. All the samples tested did, however, exhibit antimutagenic potential. These results are in agreement with the results obtained in Chapter 3 of this thesis as in all cases the unfermented Rooibos had a much higher antimutagenic potential at both soluble solid concentrations than the fermented Rooibos tea. The most significant decrease in antimutagenic potential was found to occur during the fermentation stage. During fermentation various polyphenolic compounds present in the tea are oxidised (Joubert, 1996) and these oxidised products may not have the same antimutagenic potential as the unoxidised compounds. Enhancing or changing the processing of Rooibos tea may

Table 6. Percentage inhibition values for Rooibos tea samples purchased from five retailers and tested in the Ames test[a].

Sample	0.0025 g.ml^{-1}	0.0125 g.ml^{-1}
Retailer 1	6.4	49.8
Retailer 2	5.4	50.8
Retailer 3	2.5	54.4
Retailer 4	6.4	53.4
Retailer 5	7.2	54.6

[a]Results are the average of triplicates

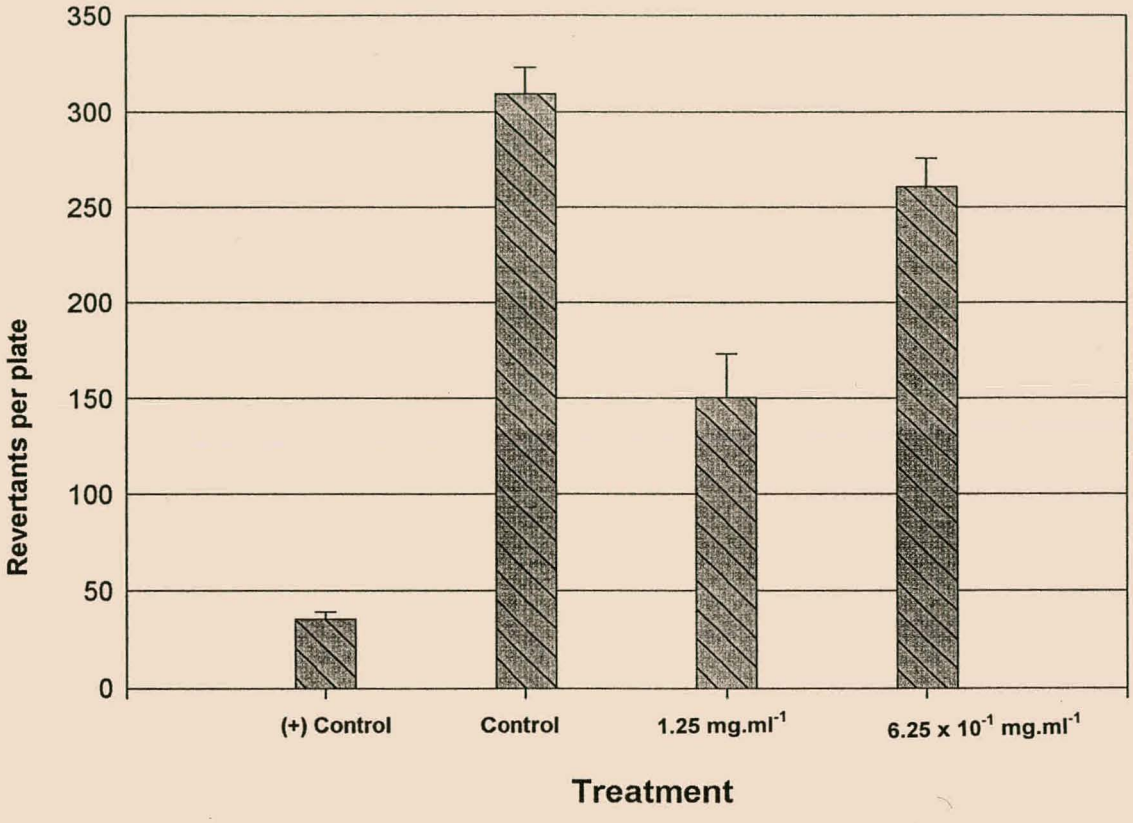

Figure 3. Antimutagenic potential of a 40% aspalathin extract. Data are averages of triplicates with error bars representing SD [Treatments: DMSO control, (+) control (2-AAF) and (+) control with crude fractions containing 40% aspalathin].

reduce the degree of oxidation which takes place, therefore affecting the antimutagenic potential, but this phenomenon must be investigated further.

The results of the Ames tests using store-purchased Rooibos tea gave similar antimutagenic potentials for all five samples tested. This was a good indication that all Rooibos tea produced has a more-or-less uniform antimutagenic potential. More samples must still be tested to obtain conclusive results. This is important in the marketing of Rooibos tea as the antimutagenic potential in the tea could, if allowed by local authorities, be advertised on the label.

The present data of a crude sample containing 40% aspalathin exhibited antimutagenic activity. However, subsequent testing of pure aspalathin has to be conducted to show whether it has protective properties against mutagenesis or not.

The identification of the fermentation step as the main cause of the decrease in antimutagenic potential in Rooibos tea is the first step in optimising the manufacturing process to produce Rooibos tea with enhanced antimutagenic potential.

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CHAPTER 5

DETECTION OF GENOTOXIC AND TOXIC POTENTIAL IN FERMENTED ROOIBOS, UNFERMENTED ROOIBOS, GREEN AND BLACK TEA USING THE TOXI- AND SOS-CHROMOTEST TECHNIQUES

Abstract

The SOS-Chromotest and the Toxi-Chromotest were utilised using mutant strains of *Escherichia coli* to investigate the genotoxic and toxic potential of fermented Rooibos, unfermented Rooibos, green and black tea extracts. These results would complement the Ames tests data from the previous chapters in this thesis with respect to the antimutagenic capacity of the tea extracts. The results from the SOS-Chromotest were inconclusive, as only a very faint colour reaction occurred, whereas the results from the Toxi-Chromotest suggested that all four tea types showed a toxic effect on the test bacteria. It was therefore decided to determine the influence of the tea samples on the growth of mutant strains of *E. coli* used in the test kits.

Growth studies on the API 20E characterised mutant *E. coli* strains and a control *E. coli* (ATCC 58) strain were determined over a period of 12 h in MRS-medium in the presence and absence of the various tea samples. Two concentrations, based on the tea's soluble solid content, were used (12.5 and 2.5 $mg.ml^{-1}$). The resulting growth studies showed that all four tea samples negatively influenced the growth of all three of the *E. coli* strains when compared to the controls. The fermented and unfermented Rooibos samples inhibited the growth of the bacteria more effectively than the green and black tea samples at both soluble solid concentrations used (12.5 and 2.5 $mg.ml^{-1}$).

Introduction

The genotoxic and toxic influence of a substance are of extreme importance in the food industry where the production and consumption of safe and healthy foods are increasingly being demanded by the general public in the interest of good health.

In previous research (Chapter 3) it was found that fermented and unfermented Rooibos as well as green and black tea extracts exhibited different levels of antimutagenic potential as shown using the Ames test. It was thus decided to further investigate and determine the genotoxicity and toxicity of the four tea extracts using the SOS-Chromotest and Toxi-Chromotest respectively. These tests are efficient in determining toxicity and genotoxicity of samples as they produce results in only a few hours and the tests are also sensitive to low levels of toxicants or genotoxicants.

The SOS-Chromotest measures the primary response of a prokaryotic cell to genotoxic damage and the test relies on a mutant strain of *Escherichia coli* (Fish *et al.*, 1987). An unrelated gene for the enzyme β-galactosidase, normally absent from this bacterium, is linked to the operator gene "SOS". The operator gene is activated whenever a genotoxic agent causes a lesion to the DNA. When activated, the SOS gene normally triggers a DNA "repair" system. With the SOS-Chromotest strain, the SOS system is activated by genotoxic assault, and results in the production of the enzyme β-galactosidase. The reaction of β-galactosidase with a chromogenic substrate results in a colour reaction from which results can be determined (Quillardet & Hofnung, 1993).

The SOS-Chromotest has often been applied in the food industry for the detection of beverage or food genotoxic contaminations and is important in ensuring the safety of foods or food components. Examples include the detection of aflatoxin B1 in orange juice or cow milk (Quillardet & Hofnung, 1993) and chlorinated butenoic acids in chlorinated drinking water (Tinkanen & Kronberg, 1990). The monitoring of natural genotoxic compounds in smoked foods in the presence of nitrite under acidic conditions has also been performed (Quillardet & Hofnung, 1993).

Similarly, the Toxi-Chromotest has been used to detect toxicants in water, chemicals, pharmaceuticals, foodstuff, food additives and cosmetics (Reinhartz *et al.*, 1987; Kwan & Dutka, 1992). The Toxi-Chromotest, in contrast, is based on the ability of toxic materials to inhibit *de novo* the synthesis of the inducible enzyme β-galactosidase in a rough mutant strain of *E. coli.* The sensitivity of the Toxi-Chromotest is enhanced by exposing the

bacteria to stressing conditions (Reinhartz *et al.*, 1987). The stressed bacteria are mixed with a cocktail containing essential factors required for the recovery of the bacteria from the stress. The activity of the enzyme is detected by introducing a chromogenic substrate, resulting in a detectable colour formation. If toxic materials are present they interfere with the recovery of most metabolic functions and thus with the synthesis of the enzyme, resulting in a decreased colour formation.

The aim of this study was to determine the presence of genotoxic and toxic properties in water soluble extracts of fermented Rooibos, unfermented Rooibos, green and black teas using the SOS-Chromotest and Toxi-Chromotest techniques, respectively.

Materials and methods

Preparation of fermented and unfermented Rooibos, green and black tea water soluble extracts

A 2.5 g per 100 ml solution of each tea extract was prepared by adding 2.5 g freeze-dried tea extract (freeze-dried on a soluble solid basis) to 100 ml of distilled water. One hundred millilitres was centrifuged at 3 g for 30 min at room temperature. The supernatant was filtered under vacuum through a 0.45 µm acetate membrane filter (Micron Separations Inc.). This filtrate was then sterilised through a 0.22 µm acetate sterile syringe filter (Micron Separations Inc.).

The SOS-Chromotest

The SOS-Chromotest was performed according to the instructions provided by the SOS-Chromotest manufacturer (Enviromental Bio Detection Products Inc., Ontario, Canada). The test was performed three times in duplicate using the fermented Rooibos, unfermented Rooibos, green and black tea extracts.

Briefly, the standard procedure for the SOS-Chromotest consists of incubating a logarithmic phase culture of the supplied SOS bacterial strain with the tea extract (0.1 ml) for 2 h at 37°C. In response to the DNA damage

inflicted by the test compound, the SOS (=DNA repair) system is activated, including the *sfiA* promoter. Since the *sfiA* promoter is fused to the structural *lacZ* gene, β-galactosidase is induced and synthesised as a reponse to the DNA damaging activity. The amount of β-galactosidase formed is then estimated by the use of *o*-nitro-phenyl-β-d-galactoside (ONPG = a chromogenic substrate of the enzyme). The amount of colour produced is a direct measure of the genotoxic damage to the DNA of the SOS-Chromotest genetically engineered *E. coli* bacterial strain. Therefore, the more genotoxic a sample is, the darker the colour reaction (Quillardet & Hofnung, 1993). A positive control (2-amino-anthracene) is included in the assay.

The Toxi-Chromotest

The Toxi-Chromotest was performed according to the instructions provided by the Toxi-Chromotest kit (version 3.0, Environmental Bio Detection Products Inc., Ontario, Canada). The test was performed three times in duplicate using the fermented Rooibos, unfermented Rooibos, green and black tea extracts.

The Toxi-Chromotest procedure consists of incubating the logarithmic phase culture of the supplied pre-stressed Toxi-Chromotest bacterial strain with the tea extract (0.1 ml) and a cocktail, containing a specific inducer of β-galactosidase and specific growth factors essential for the recovery of the bacteria from their stressed condition, for 2 h at 37°C. The activity of the induced enzyme is detected by the hydrolysis of a chromogenic substrate. Toxic substances interfere with the recovery process of the pre-stressed bacterial strain and thus with the synthesis of the enzyme and the resultant colour reaction. Therefore, the more toxic a sample is, the lighter the colour reaction will be. A positive control (mercury chloride) is also included in the assay.

Strain characterisation and identification

A control *Escherichia coli* strain (American Type Culture Collection strain 58) and the two mutant SOS-Chromotest and Toxi-Chromotest bacterial strains were characterised according to the API 20E system (bioMérieux sa, 69280 Marcy l'Etoile, France). The morphology of each culture was determined by

bright field microscopy of Gram-stained preparations. The following tests were also performed: catalase, oxidase, endospore formation and growth on MacConkey agar (Gerhardt *et al.*, 1981).

Growth studies

The cultures used in the growth studies were the standard *Escherichia coli* (ATCC 58) and the mutant SOS-Chromotest and Toxi-Chromotest bacterial strains. The cultures were initially cultivated in MRS-medium (normal-MRS) with the pH set at 7.0 before sterilisation (Merck). From this a 2% (v/v) inoculum (equivalent to McFarland Standard 2 = bacterial concentration of 6.0 x 10^8) of each test organism was inoculated into 1 l MRS-medium (tea-MRS: prepared with the tea extract in place of the distilled water) and incubated at 37°C throughout the experiment. Two soluble solid concentrations (2.5 and 12.5 mg.ml^{-1}) of each tea extract were tested. At one hour intervals, the inoculated cultures were shaken gently and 1 ml samples taken for serial dilutions in Ringers solution (10^{-1} to 10^{-8}). The optical density (OD_{600}), using a Spectronic 20 (Bausch & Lomb), was monitored. Controls where the three strains were grown in normal MRS-medium (without tea extract), were also evaluated.

Results and discussion

The antimutagenic potential of fermented and unfermented Rooibos tea as well as green and black tea has already been confirmed (Chapters 3 and 4 of this thesis). The SOS-Chromotest and Toxi-Chromotest were thus performed to investigate the genotoxic and toxic effects of the same tea extracts as this would reveal any genotoxic or toxic potential in the tea.

SOS-Chromotest

The results (triplicate experiments performed in duplicate) (Fig. 1) of the SOS-Chromotest using the four tea extracts in an increasing dilution series (12.5 mg.ml^{-1} in row A to 0.0125 x 10^{-4} mg.ml^{-1} in row H) produced only

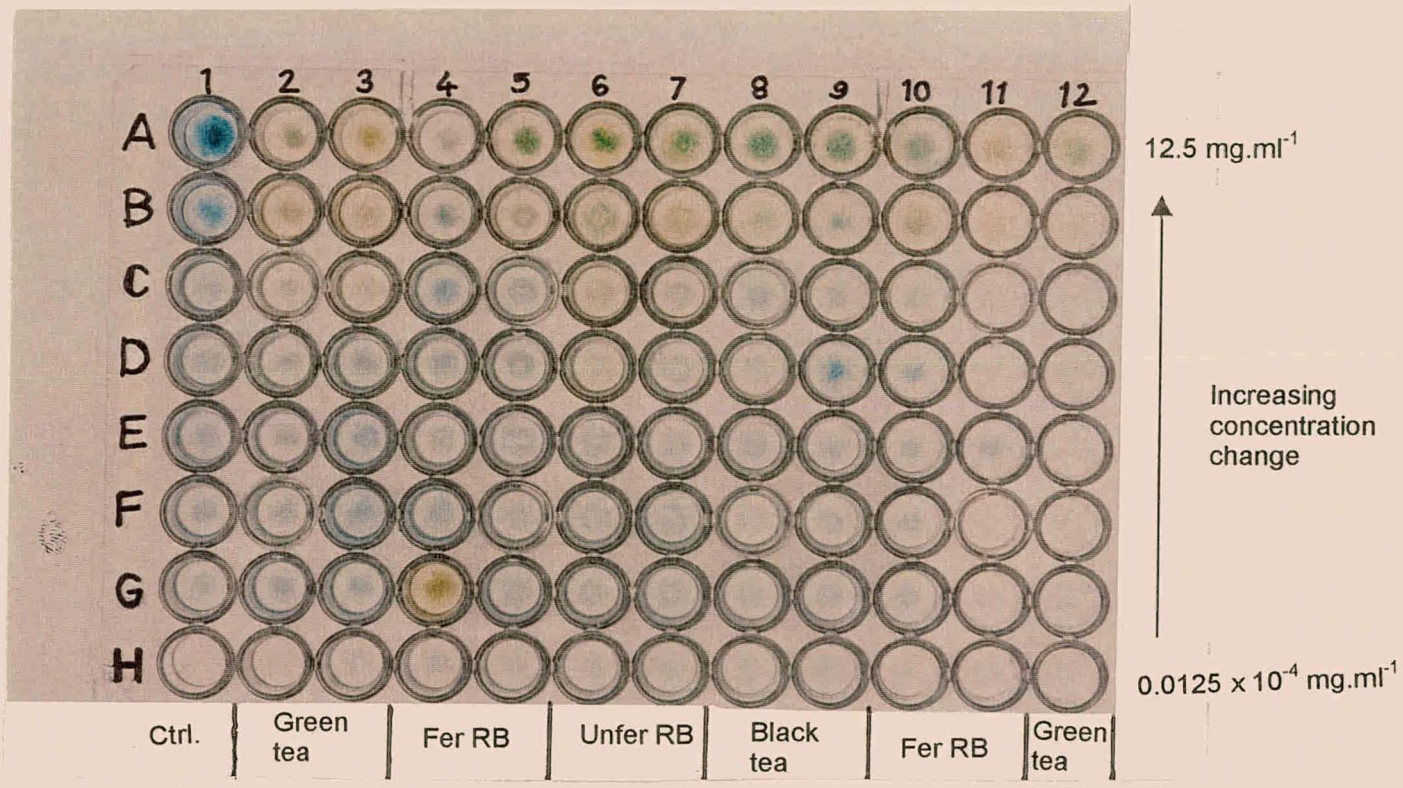

Figure 1. Data showing the colour scale results of the SOS-Chromotest using fermented Rooibos (Fer RB), unfermented Rooibos (Unfer RB), green (Green) and black (Black tea) ([a]Control: mutagen 2-amino-anthracene).

a very faint colour reaction for all the repeats from which no specific conclusions could be made regarding the genotoxic effect of the tea extracts on the genetically engineered *E. coli* test strain (Fig. 1). The control mutagen used was 2-amino-anthracene and this produced a clear decrease in colour density as the concentration (Fig. 1 = column 1) decreased (Environmental Bio Detection Products Inc.,Ontario, Canada), indicating that the SOS-Chromotest bacteria were functioning properly. No clear repeatable colour trend could be identified from the colour results (Fig. 1) using the four tea extracts even as the tea extracts were diluted (high concentrations may not induce any response due to high toxicity according to the manufacturers instructions). The yellow-brown colour identified in columns 2 to 12 and rows A to C of Fig. 1 indicated where the tea concentration was very high and masked the colour of the indicator. These inconclusive results suggested that the SOS-Chromotest was not as efficient for determining genotoxicity in tea as was previously thought. However, it is possible that the inconclusive colour profiles could be as a result of the tea extracts inhibiting the actual growth of the test bacteria and thus directly influencing the colour change.

Toxi-Chromotest

The colour profiles of the Toxi-Chromotest indicated that a definite colour reaction had occurred (Columns 2 to 12 of Fig. 2). The control, (Fig 2. = column 2) where the toxin mercury chloride was included, produced a definite increase in colour density as the concentration of the mutagen decreased, therefore indicating toxic activity. The yellow-brown colour evident in columns 3 to 12 and rows A to D (Fig. 2) of the microtiter plate indicated where the tea concentration was very high and masked the colour of the indicator. The results using the four tea extracts indicated a gradual increase of colour concentration as the concentration of the teas decreased. The data also showed that the fermented and unfermented Rooibos tea exhibited a decreasing effect on the mutant *E. coli* test strain as the tea was diluted. In the case of the green and black teas, the colour profiles indicated a much more intense effect. Tea is a universally consumed beverage and it is thus unlikely that the tea had a toxic effect on the bacteria. It was, however,

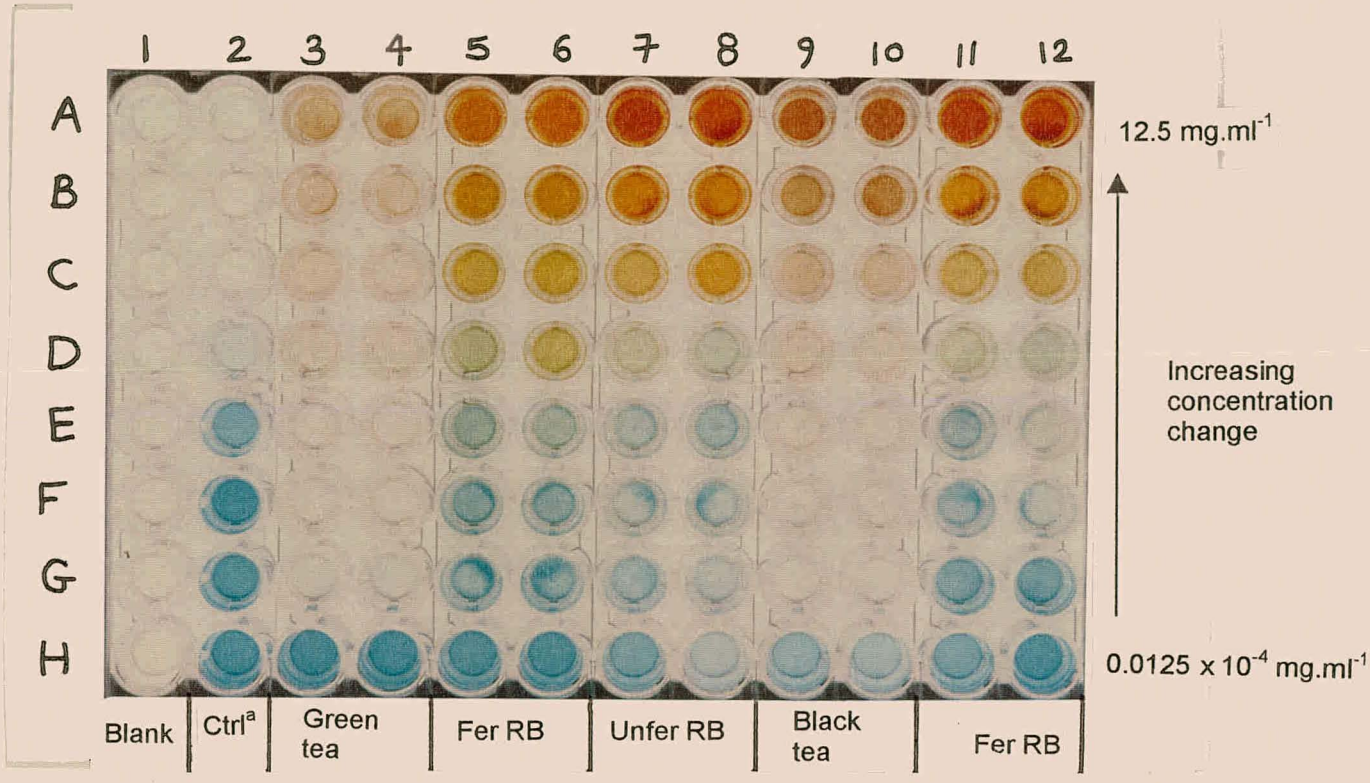

Figure 2. Data showing the colour scale results of the Toxi-Chromotest using fermented Rooibos (Fer RB), unfermented Rooibos (Unfer RB), green (Green) and black (Black) tea ([a]Control: Standard toxin – 4 $\mu g.ml^{-1}$ mercury chloride).

thought possible that the tea may rather be inhibiting the growth of the test bacteria and thus preventing the correct development of the colour changes.

The colour profiles obtained in the case of the SOS-Chromotest were considered to be unreliable as they were unclear and not reproducible. Similar variations, but not so pronounced in colour reaction, were also found for the Toxi-Chromotest. It was thus concluded that this may not be as a result of toxicity of the tea extracts but rather a general influence that the tea had on the growth of the test bacteria. It was therefore decided to use a control *E. coli* strain (ATCC 58) as well as the test strains from the SOS-Chromotest and Toxi-Chromotest and to determine if the four tea extracts really influenced the growth of the bacteria. The bacteria were first characterised to confirm their identification.

Characterisation and identification

A control *Escherichia coli* strain (ATCC 58) and the mutant SOS-Chromotest and Toxi-Chromotest *E. coli* strains were characterised using the general tests and the API 20E system (Table 1). The results (Table 1) when applied to the API Identification Data Base confirmed the identity of the control bacteria as an *E. coli* strain (ID% = 99%). The mutant SOS-Chromotest and Toxi-Chromotest bacterial strains differed greatly from the control *E. coli* strain (ATCC 58) and could not be identified as *E. coli* strains (Table 2) using the API 20E system.

Standard growth studies

Growth studies using the three bacterial strains (control *E. coli* and the mutant SOS-Chromotest and Toxi-Chromotest strains) were carried out to determine the growth pattern of each of the strains while being incubated in the presence of the four tea extracts.

Firstly, as a set of controls, the growth of the three bacterial strains was evaluated in normal MRS-medium prepared with distilled water (Fig. 3 - 5). Growth of the three strains was then evaluated with tea-MRS-medium prepared with the two soluble solid concentrations (2.5 and 12.5 $mg.ml^{-1}$) of each of fermented Rooibos, unfermented Rooibos, green and the black tea extracts (Fig. 6 - 11). The growth of the bacteria in normal-MRS-medium and

Table 1. Characteristics of the mutant bacteria from the SOS-Chromotest and Toxi-Chromotest kits and a control *E. coli* strain (ATCC 58).

Test	*E. coli* (ATCC 58)	Mutant *E. coli* from the Toxi-Chromotest	Mutant *E. coli* from the SOS-Chromotest
Gram	-	-	-
Endospore	-	-	-
Catalase	-	-	-
Motility	+	+	+
Morphology	cocci	cocci	cocci
Growth on MacConkey	+	+	+
API 20E			
ONPG – beta galactosidase	+	-	-
Arginine dihydrolase	+	+	+
Lysine decarboxylase	+	-	-
Orthinine decarboxylase	+	-	-
Citrate utilisation	-	-	-
H_2S production	-	-	-
Urease	-	+	+
Tryptophane deaminase	-	-	-
Indole production	+	-	-
Acetoin production	-	+	+
Gelatinase	-	-	+
Fermentation of:			
Glucose	+	-	-
Mannitol	+	+	-

Table 1. Continued

Inositol	-	-	-
Sorbitol	+	-	-
Rhamnose	+	-	-
Sucrose	-	-	-
Melibiose	+	-	-
Amygdalin	-	-	-
Arabinose	+	-	-
Cytochrome-oxidase	-	+	+
NO_2 production	+	+	+
Reduction to N_2 gas	-	-	-

ONPG: ortho-nitro-phenyl-galactoside

Table 2. Identification of bacteria used in API 20E tests.

Strain	ID% on API 20E
ATCC 58	99% - *E. coli*
SOS-Chromotest mutant	No identification
Toxi-Chromotest mutant	No identification

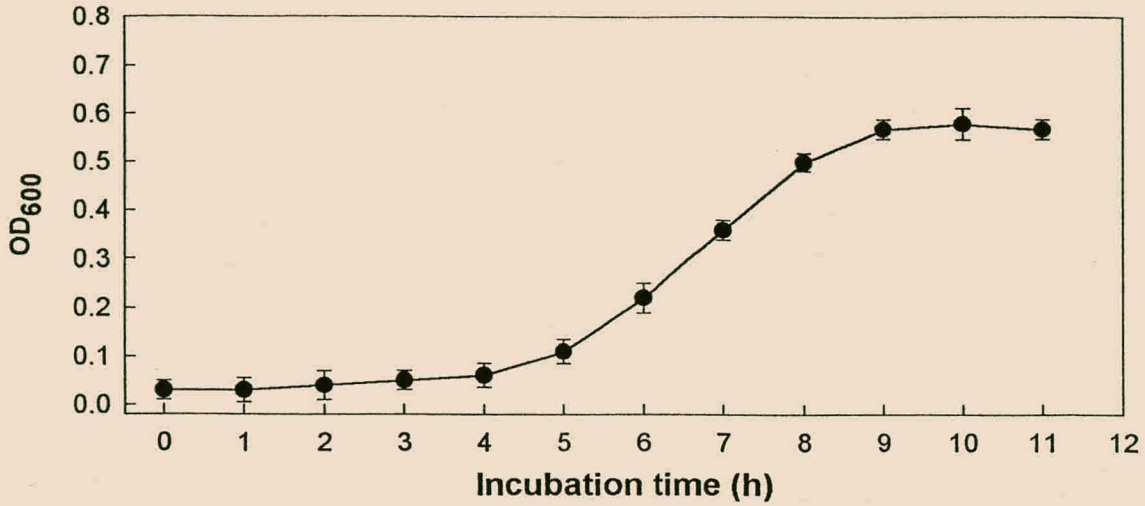

Figure 3: Growth curve of the *E. coli* strain (ATCC 58) in normal-MRS with error bars representing standard deviation of four repeats.

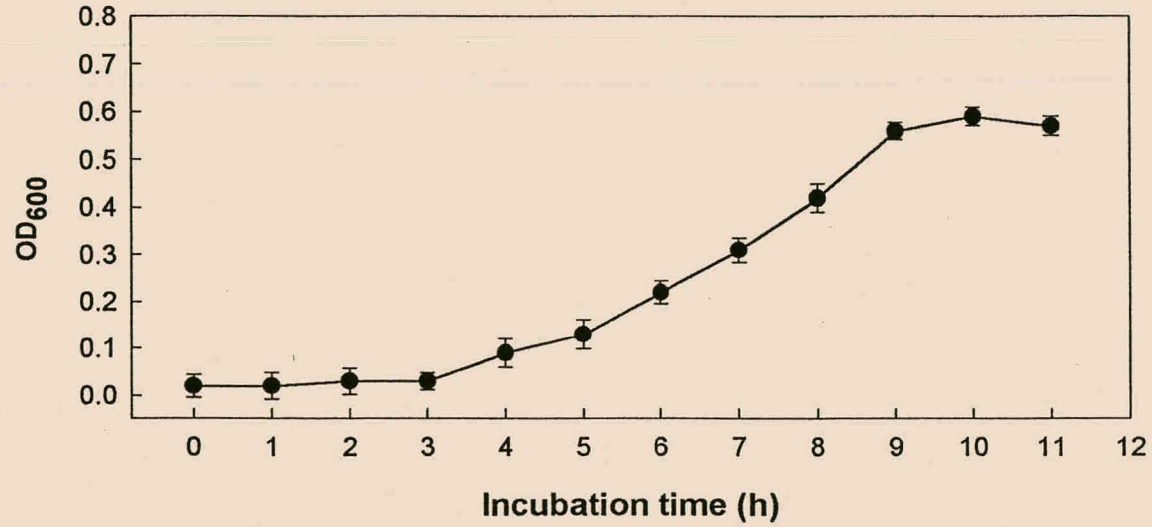

Figure 4: Growth curve of the mutant *E. coli* strain from the SOS-Chromotest in normal-MRS with error bars representing standard deviation of four repeats.

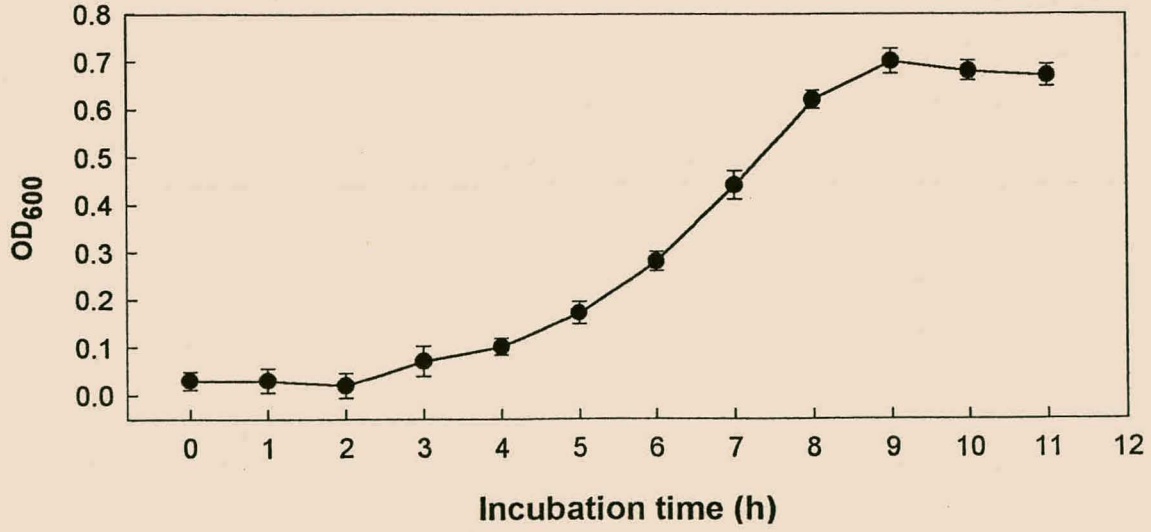

Figure 5: Growth curve of the mutant *E. coli* strain from the Toxi-Chromotest in normal-MRS with error bars representing standard deviation of four repeats.

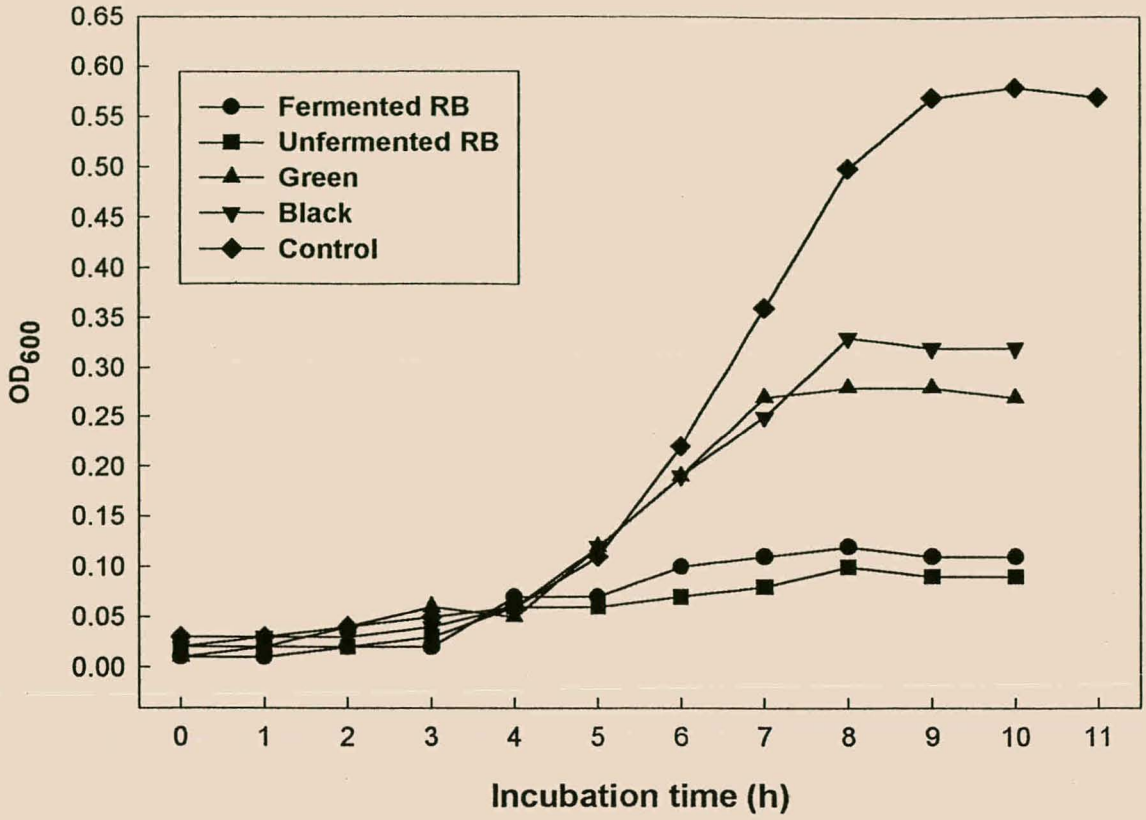

Figure 6: Growth curve of the *E. coli* (ATCC 58) strain cultured in tea-MRS with four different tea extracts at a soluble solid concentration of 12.5 mg.ml^{-1} (Control: normal-MRS prepared without tea extract but distilled water).

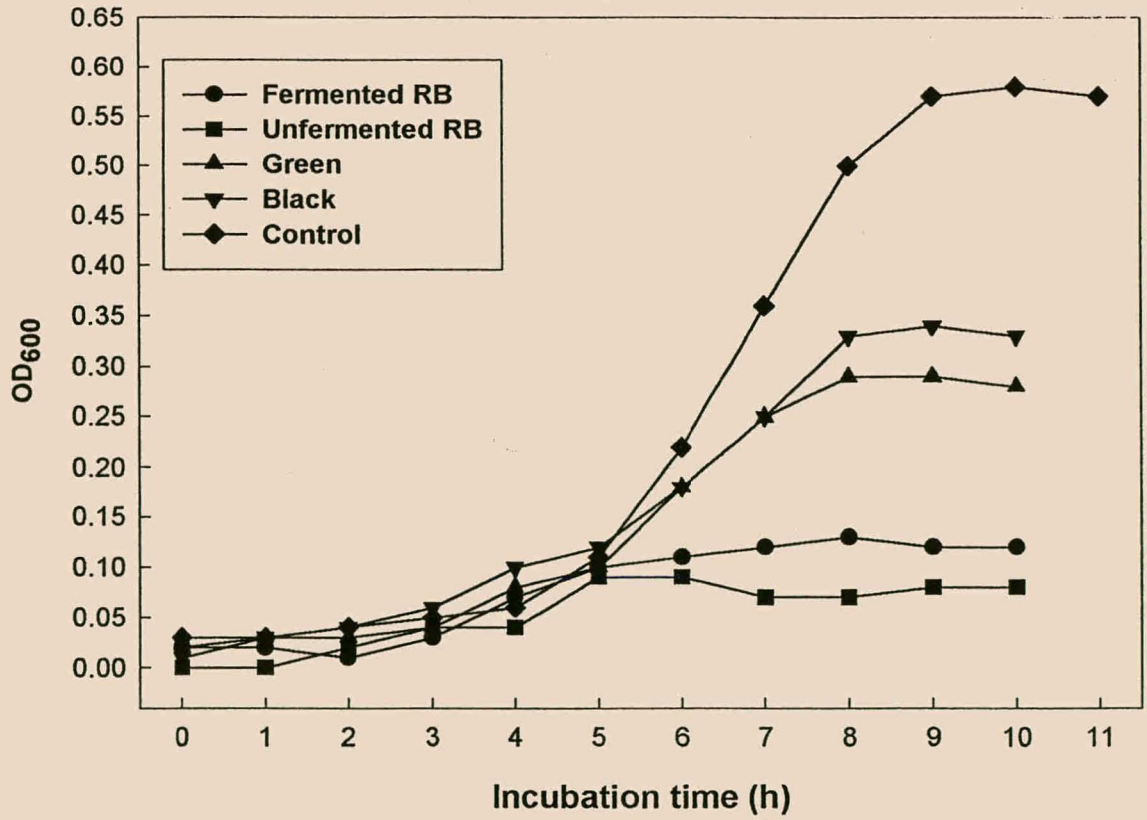

Figure 7: Growth curve of the *E.coli* (ATCC 58) strain incubated in tea-MRS with four different tea extracts at a soluble solid concentration of 2.5 mg.ml^{-1} (Control: normal-MRS prepared without tea extract but distilled water).

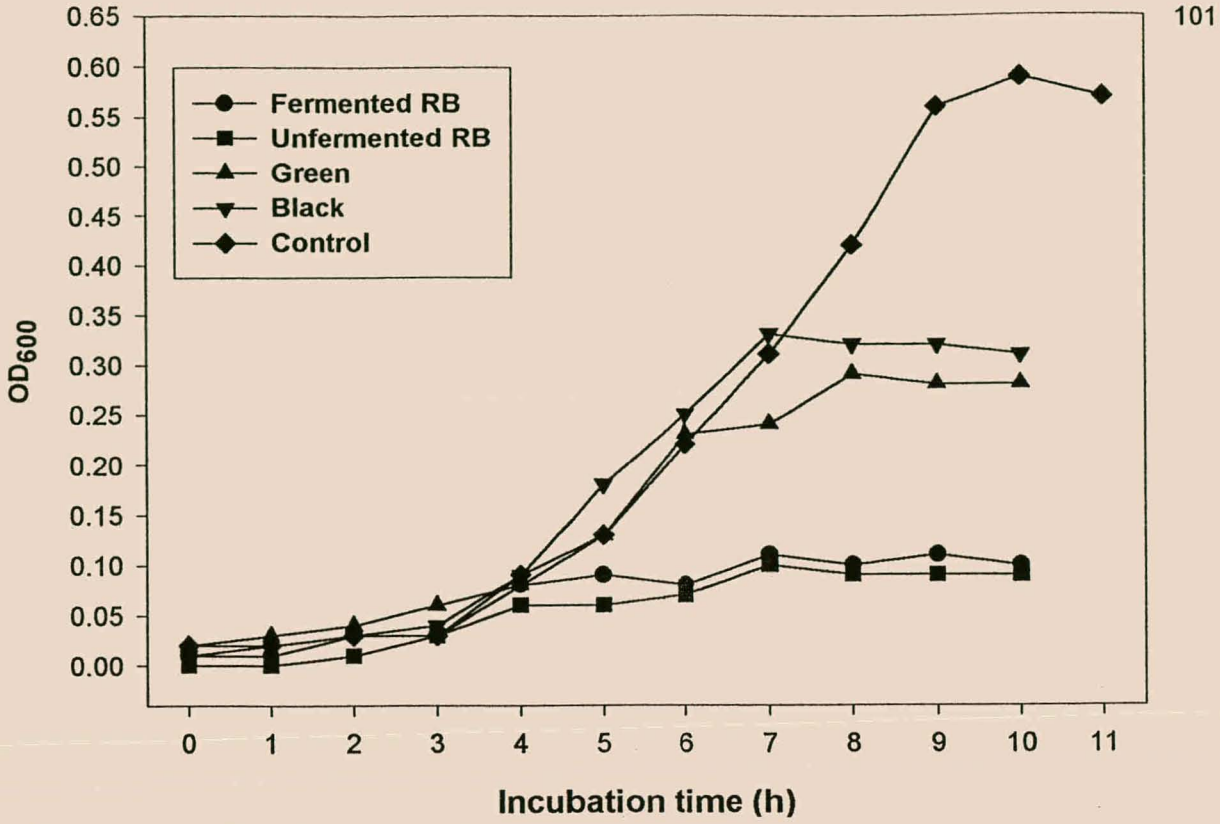

Figure 8: Growth curves of a mutant *E. coli* strain from the SOS-Chromotest incubated in tea-MRS with four different tea extracts at a soluble solid concentration of 12.5 $mg.ml^{-1}$ (Control: normal-MRS prepared without tea extract but distilled water).

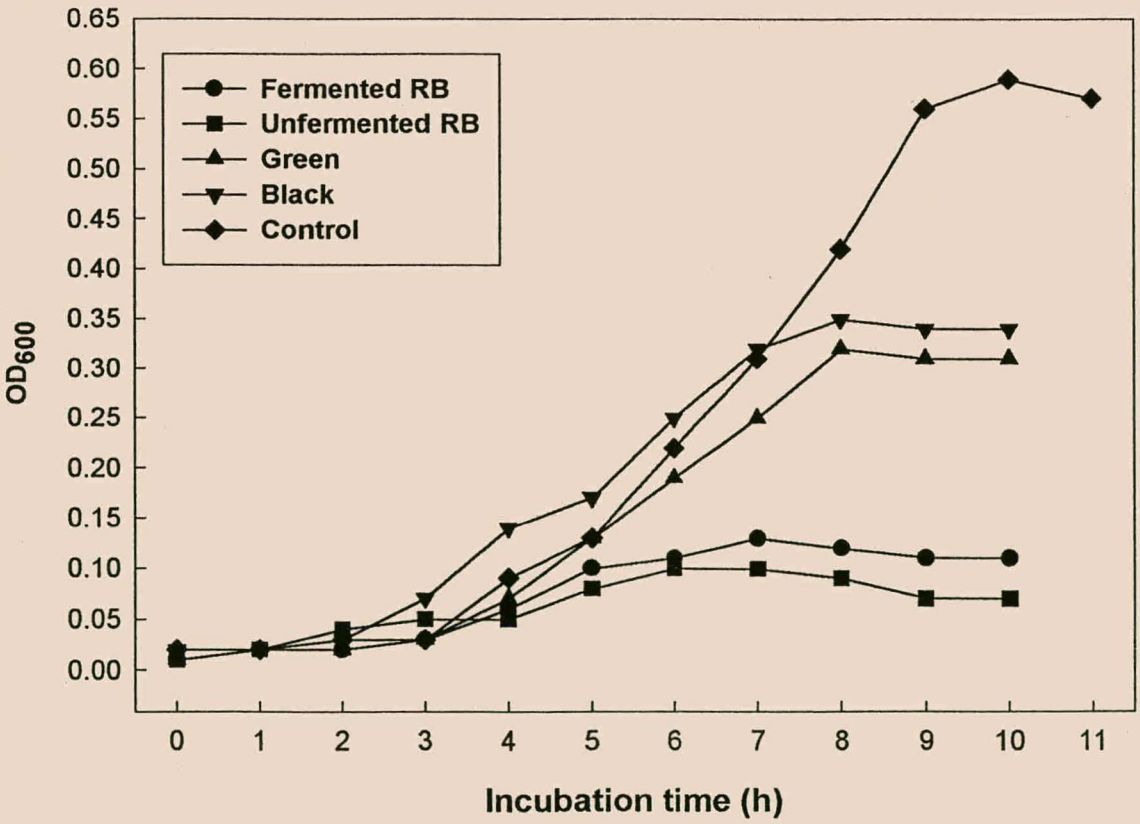

Figure 9: Growth curves of a mutant *E. coli* strain from the SOS-Chromotest incubated in tea-MRS with four different tea extracts at a soluble solid concentration of 2.5 $mg.ml^{-1}$ (Control: normal-MRS prepared without tea extract but distilled water).

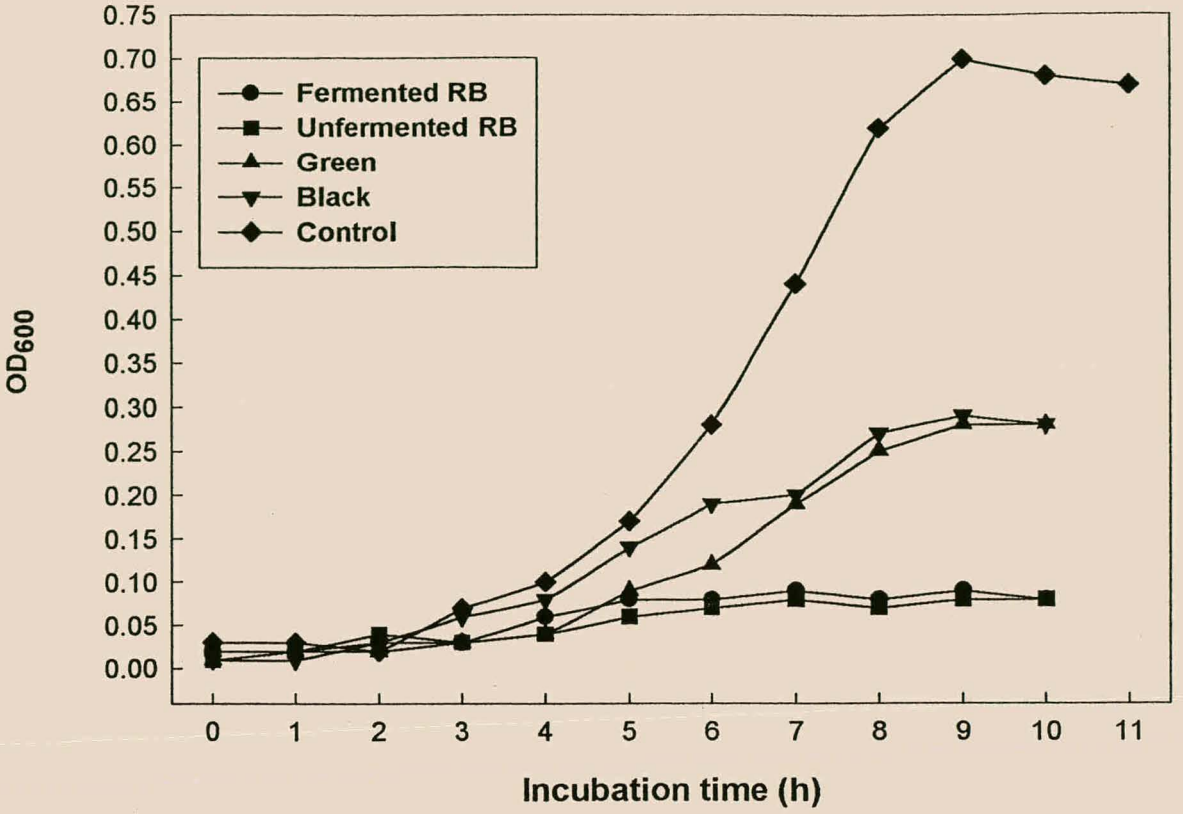

Figure 10: Growth curves of a mutant *E. coli* strain from the Toxi-Chromotest incubated in tea-MRS with four different tea extracts at a soluble solid concentration of 12.5 $mg.ml^{-1}$ (Control: normal-MRS prepared without tea extract but distilled water).

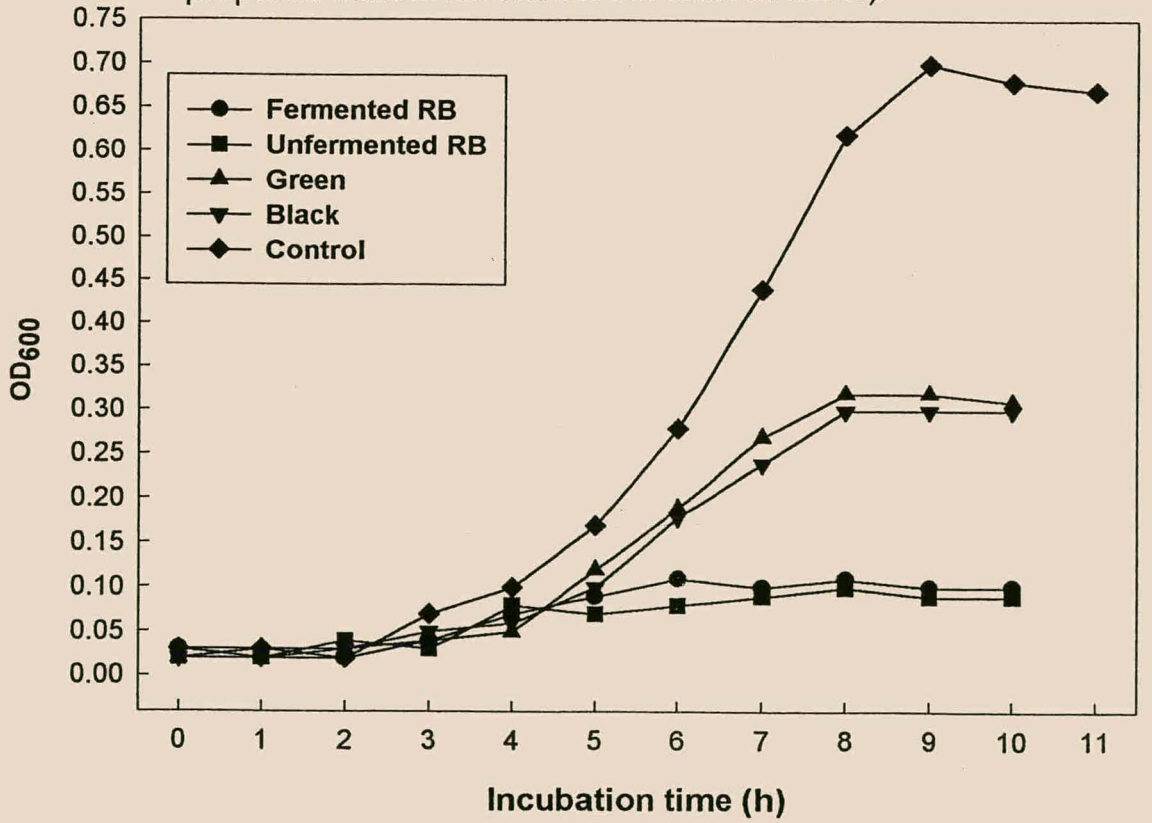

Figure 11: Growth curves of a mutant *E. coli* strain from the Toxi-Chromotest incubated in tea-MRS with four different tea extracts at a soluble solid concentration of 2.5 $mg.ml^{-1}$ (Control: normal-MRS prepared without tea extract but distilled water).

the tea-MRS-medium at 37°C for 12 h was monitored to determine if the tea extracts were really affecting the growth of the bacteria. The soluble solid concentrations used in this study were chosen to correspond to those used in the SOS-Chromotest and Toxi-Chromotest as well as the concentrations used in the previous Chapters (3 and 4) of this thesis.

The data obtained (Fig. 3 – 11) clearly showed that all the tea extracts inhibited the growth of the three *E. coli* strains at both tea soluble solid concentrations used (2.5 and 12.5 $mg.ml^{-1}$) when compared to the controls. The results also showed that the fermented and unfermented Rooibos tea had a much stronger inhibitory effect on the growth of the three strains than the green and black teas at both tea concentrations (Fig. 6–11). The strong inhibitory influences that the tea extracts had on the bacterial strains are summarised in Tables 3 and 4.

Conclusions

The results from the Toxi-Chromotest showed that a colour reaction had taken place when the fermented and unfermented Rooibos tea extracts were evaluated for toxicity. A similar, but less obvious, colour reaction has taken place when evaluating the green and black tea extracts. From this it was concluded that the tea extracts had a toxic effect on the Toxi-Chromotest test bacterium. Since tea is a universally consumed beverage it was suspected that the different teas may rather have had an inhibitory effect on the growth of the bacterium and not a toxic reaction to cause this reaction. In contrast, the results from the SOS-Chromotest revealed a very faint colour reaction from which no specific conclusions could be made. It was again suspected, as with the Toxi-Chromotest, that all four teas may rather have had an inhibitory effect on the growth of the SOS-Chromotest bacterium resulting in the weak reaction profiles.

To explain the inconclusive results obtained with the SOS-Chromotest and Toxi-Chromotest growth studies were performed and confirmed that all four tea extracts did inhibit the growth of the bacterial test strains used in both the SOS-Chromotest and Toxi-Chromotest. A control *E. coli* strain (ATCC 58)

Table 3. Averaged percentage inhibition values[a] in tea MRS-medium for three *E. coli* strains incubated with fermented Rooibos (RB), unfermented Rooibos, green and black teas at a soluble solid concentration of 12.5 $mg.ml^{-1}$. Growth in normal-MRS was used as the control.

Tea extract	ATCC 58 (%)	Mutant SOS-Chromotest bacterium (%)	Mutant Toxi-Chromotest bacterium (%)
Fermented RB	78.6	80.1	85.4
Unfermented RB	83.9	84.5	85.6
Green	50.1	51.8	56.8
Black	41.1	46.6	56.8

[a]Percentage inhibition = 100 - [Final OD_{600}*/ Final OD_{600} (control) x 100]

*OD_{600} = Optical Density at 600 nm

Table 4. Averaged percentage inhibition values[a] in tea MRS-medium for three *E. coli* strains incubated with fermented Rooibos (RB), unfermented Rooibos, green and black teas at a soluble solid concentration of 2.5 $mg.ml^{-1}$. Growth in normal-MRS was used as the control.

Tea extract	ATCC 58 (%)	Mutant SOS-Chromotest bacterium (%)	Mutant Toxi-Chromotest bacterium (%)
Fermented RB	77.8	78.5	79.5
Unfermented RB	82.5	82.3	80.2
Green	48.5	47.1	51.2
Black	40.2	42.1	52.0

[a]Percentage inhibition = 100 - [Final OD_{600}*/ Final OD_{600} (control) x 100]

*OD_{600} = Optical Density at 600 nm

was included to confirm that the teas also inhibited the growth of a non-mutant *E. coli* strain.

The results from the growth studies clearly showed that fermented and unfermented Rooibos tea had a stronger inhibitory effect on the growth of the bacteria than the green and the black tea extracts and thus possibly had antibacterial activity. These results suggest that the limited growth of the bacterial strains may have prevented the bacteria from producing the enzyme β-galactosidase, essential in the development of the colour reaction in both the SOS-Chromotest and Toxi-Chromotest. The ability of the tea extracts to inhibit *E. coli* bacterial growth is an important characteristic of the tea and must be investigated in the future.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Rooibos tea (*Aspalathus linearis*) is cultivated and processed in the Clanwilliam area of the Western Cape and has become increasingly popular over the past decade due to its alleged health properties. Rooibos tea is alleged to cure insomnia, allergies and nervous complaints (Joubert & Ferreira, 1996) and it is also considered an anti-aging beverage by the Japanese (Yen & Chen, 1995). The confirmation of antimutagenic potential in Rooibos tea is important in the fight for cancer prevention and further research on the chemo-preventive properties of Rooibos tea would substantiate it as a health promoting agent.

One of the best documented and researched bacterial tests to illustrate mutagenicity is the *Salmonella typhimurium* mutagenicity (Ames) assay (Maron & Ames, 1983) which relies on the reaction of one of four specific *Salmonella typhimurium* strains to a test compound. The Ames test is designed as a simple and rapid method to demonstrate antimutagenic potential. In this study it was clearly shown, using the Ames test, that unfermented Rooibos as well as fermented Rooibos tea possess antimutagenic properties. This is, as far as is known, the first time that Rooibos tea has been shown to have antimutagenic activity.

In this study, green and black teas were included as controls to compare to the antimutagenic potential of Rooibos tea. The green tea proved to have the highest antimutagenic potential, ca. 15% stronger than that of the unfermented Rooibos tea. However, in this study only one batch of green and black tea was tested in the Ames assays. The antimutagenic potential of different batches of each tea type must still be evaluated. It was also found that the two processed teas (black and fermented Rooibos tea) had similar but much lower antimutagenic potentials than the unfermented (unprocessed) Rooibos and green teas. The fact that both the unfermented teas had much stronger antimutagenic potentials than the processed teas suggested that the processing of the black and fermented Rooibos tea's may have led to a decrease in antimutagenic potential of the teas.

This hypothesis, that the processing of Rooibos tea resulted in tea with a decreased antimutagenic potential, was confirmed when randomly collected

samples of Rooibos tea from the five major processing stages (unfermented Rooibos tea, fermented tea, fermented sun-dried tea, tea from before steam pasteurisation and tea from after steam pasteurisation) were tested in the Ames test. In all cases the unfermented Rooibos tea showed a stronger antimutagenic potential than the fermented Rooibos teas from the different processing stages. Statistical analyses confirmed that it was the fermentation process that caused the significant decrease in antimutagenic potential. Processing results in the oxidation of various polyphenolic compounds (Joubert, 1996) present and this would probably lead to a decrease in antimutagenic potential of the teas. Optimizing the different processing stages of Rooibos tea to reduce the oxidation that takes place may minimize the loss of antimutagenic potential. This would be important in maximising the chemo-preventive properties in the tea.

Investigation into the antimutagenic potential of the four teas was done at five concentrations and the results clearly indicated a dose-response curve where, the higher the concentration of tea, the stronger the resulting antimutagenic potential. This was an indication that the tea samples did not have a toxic effect on the Ames tester strains used (Dr. W.C.A. Gelderblom, 1999, Medical Research Center, Tygerberg, personal communication). However, further investigation must be done to confirm this phenomenon and determine whether an optimum concentration for mutation inhibition (antimutagenic potential) can be attained.

Investigation of Rooibos tea samples is important to determine if the antimutagenic potential identified is common to Rooibos tea sold on the retail market. To determine this, random tea samples from five commercial retailers were obtained. It was found that the antimutagenic potential in all these Rooibos tea samples was very similar and the results confirmed the antimutagenic potential identified in the processed Rooibos tea in the first study. This was a good indication that the processing of Rooibos tea leads to a fairly uniform antimutagenic activity. This is important in the marketing of Rooibos tea as the antimutagenic potential in the tea can, where allowed by the local authorities, then be advertised on the label. Consumer awareness as to the benefits of Rooibos tea will also be improved.

Polyphenols are thought to be the components responsible for the antimutagenic activity in green tea (Yen & Chen, 1996). Identification of Rooibos tea polyphenols as having antimutagenic potential could lead to the purification of

these compounds and their use as chemo-preventive agents. Since aspalathin is one of the major polyphenols found in Rooibos tea (Joubert & Ferreira, 1996), the Ames test was performed using a crude aspalathin fraction to determine if polyphenols or more specifically aspalathin, are responsible for the antimutagenic properties in the Rooibos tea. The Ames test, incorporating the aspalathin rich fraction, revealed that the crude fraction had antimutagenic properties that, on average, were 5 - 15% lower when compared to the antimutagenic properties in Rooibos tea water extracts. It is important that the actual antimutagenic potential of a pure aspalathin sample be determined so as to determine whether the aspalathin, has antimutagenic properties, or whether it merely enhances the antimutagenic properties already present in Rooibos tea.

The antimutagenicity of two types of Rooibos tea extracts, water-soluble and ethyl acetate soluble extract, were also determined to obtain an indication as to where the compounds responsible for antimutagenicity can be found. The ethyl acetate extract proved to have the more potent antimutagenic potential in all four of the tea samples (fermented Rooibos, unfermented Rooibos, green and black tea) tested. The fact that polyphenols, thought to be responsible for the antimutagenic potential in teas, are soluble in ethyl acetate is a good indication that polyphenols are responsible for the antimutagenic potential. Rooibos tea is rich in polyphenols, the importance of which is emphasised by the fact that polyphenols are included in the "designer" food program of the National Cancer Institute (USA) (Joubert & Ferreira, 1996). This phenomenon must however be investigated further to identify the exact compounds responsible for the tea's antimutagenicity.

Tests to identify any genotoxic and toxic properties in the four tea extracts (Quillardet & Hofnung, 1993; Reinhartz *et al.*, 1987) were performed using the SOS-Chromotest and Toxi-Chromotest. These tests are similar to the Ames test in that they are short-term tests reliant on mutant bacterial strains. Results, however, are dependent on a colour reaction, which is dependent on the production of the enzyme β-galactosidase. The SOS-Chromotest revealed a very faint but not reliable colour profile, from which no conclusions could be made, whereas the Toxi-Chromotest colour reaction suggested that all four tea types had a strong toxic effect on the Toxi-Chromotest mutant *Escherichia coli* strain.

Since tea is a universally consumed beverage it was decided to perform growth studies on the mutant *E. coli* strains from the SOS-Chromotest and Toxi-Chromotest and a control *E. coli* (ATCC 58) with the four tea extracts to determine if the tea extracts really had an effect on the growth of the bacterial tester strains. The data clearly showed that the four tea extracts did have an inhibitory effect on the growth. It was found that fermented and unfermented Rooibos tea had a much stronger inhibitory effect than the green and black teas. These inhibition responses by the tea on the tester strains may explain the inconclusive results obtained in the SOS-Chromotest and Toxi-Chromotest. The colour reactions, needed to determine positive or negative results, may have been influenced by the inhibition effect the tea samples had on the growth of the bacterial strains. The results of the growth studies indicated that the teas, especially Rooibos tea are effective in inhibiting standard *E. coli* growth. Further research, to determine the growth inhibiting response that the four teas may have on other bacterial strains and the effect this inhibition has on the human body, must still be conducted. Investigation into this phenomenon may result in the compounds responsible for this inhibition being purified and then incorporated into foods to inhibit bacterial or specifically *E. coli* growth.

The chemo-preventive properties of Rooibos tea discussed in this study will help to further promote it as a health-promoting beverage by the general public as well as the medical field. The ability of Rooibos tea to help prevent mutations which may lead to various types of cancer is also reason to consider Rooibos tea as a functional or designer food.

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